ORGAN REGENERATION IN ANIMALS

Recovery of Organ Regeneration Ability in Animals

L. V. POLEZHAEV

Professor, Dr. Biol. Sc.
Head, Laboratory of Experimental Morphology
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Academy of Sciences of the USSR, Moscow

This text represents considerable data obtained by Soviet researchers but little known to Western scientists. It is presented for all interested in the problems of regeneration in vertebrate animals and in the possibility of experimental recovery of lost regenerative ability of some vitally important organs and tissues in mammalia and in man.

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ORGAN REGENERATION IN ANIMALS

A Monograph in
The BANNERSTONE DIVISION *of*
AMERICAN LECTURES IN
LIVING CHEMISTRY

Edited by

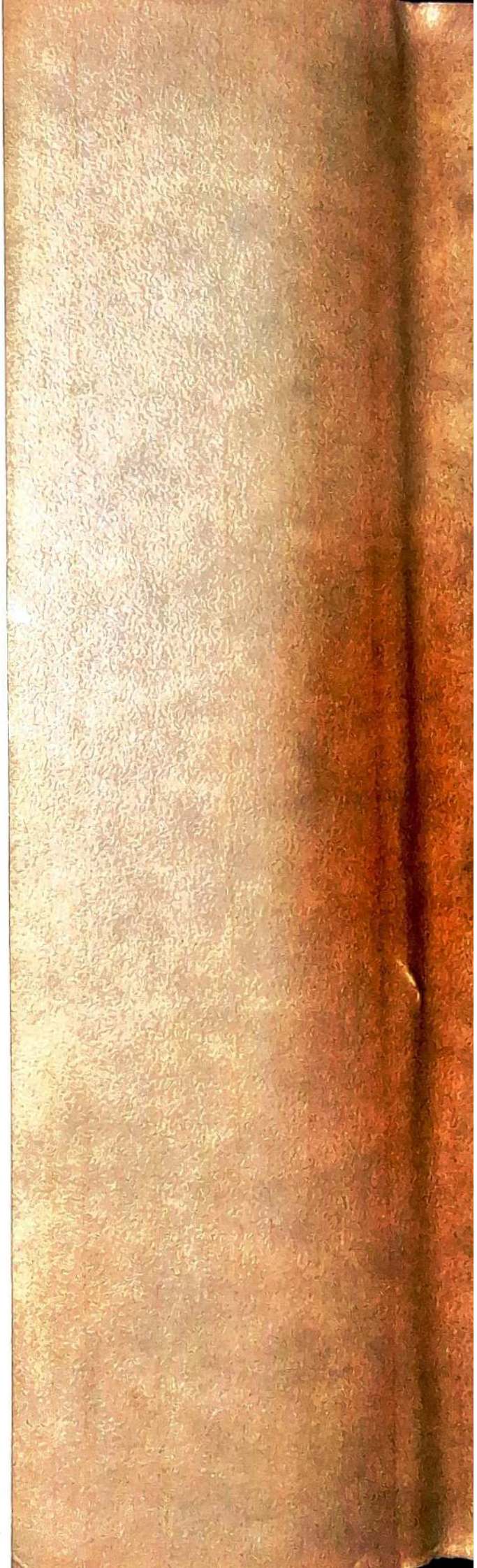
I. NEWTON KUGELMASS
M.D., Ph.D., Sc.D.
Consultant to the
Department of
Health and Hospitals
New York City

A review is given of recent experimental data, demonstrating the ability of inducing regeneration of extremities in frogs, lizards, newborn rats and opossums which do not usually regenerate after amputation.

The text is principally concerned with data on the regeneration of . . .

- *Extremities in axolotles, suppressed by the action of high doses of x-irradiation*
- *Calvaria bones in dogs and man*
- *Tooth tissue in dogs*
- *Myocardium in rats, rabbits and dogs*
- *Peripheral nerves, spinal cord and brain neurons in rats, rabbits, cats and dogs as well as some clinical data on treatment of human patients with injured spinal cord*

The author provides general principles and methods of investigation. The text is copiously illustrated and each chapter concludes with a list of the literature cited.



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BANNERSTONE HOUSE
301-327 East Lawrence Avenue
Springfield, Illinois, U.S.A.

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Publication Number 821

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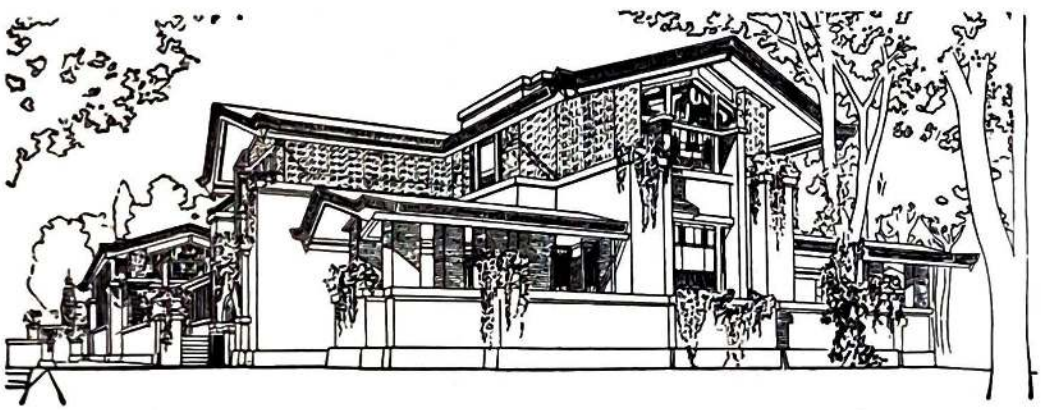

CHARLES C THOMAS • PUBLISHER
Springfield • Illinois • U.S.A.

Published and Distributed Throughout the World by
CHARLES C THOMAS • PUBLISHER
BANNERSTONE HOUSE
301-327 East Lawrence Avenue, Springfield, Illinois, U.S.A.
NATCHEZ PLANTATION HOUSE
735 North Atlantic Boulevard, Fort Lauderdale, Florida, U.S.A.

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ISBN 0-398-02381-6
Library of Congress Catalog Card Number: 75-157294

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FOREWORD

Our Living Chemistry Series was conceived by Editor and Publisher to advance the newer knowledge of chemical medicine in the cause of clinical practice. The interdependence of chemistry and medicine is so great that physicians are turning to chemistry, and chemists to medicine in order to understand the underlying basis of life processes in health and disease. Once chemical truths, proofs and convictions become sound foundations for clinical phenomena, key hybrid investigators clarify the bewildering panorama of biochemical progress for application in everyday practice, stimulation of experimental research, and extension of postgraduate instruction. Each of our monographs thus unravels the chemical mechanisms and clinical management of many diseases that have remained relatively static in the minds of medical men for three thousand years. Our new Series is charged with the *nisus élan* of chemical wisdom, supreme in choice of international authors, optimal in standards of chemical scholarship, provocative in imagination for experimental research, comprehensive in discussions of scientific medicine, and authoritative in chemical perspective of human disorders.

Dr. Polezhaev of Moscow presents unique experimental studies on the loss and recovery of regenerative ability of organs and tissues in animals that lost the regenerative capacity in their ontogenesis. Regeneration is a dynamic process while regenerative ability is a static product; the former is metabolic dependent on molecular cell structure, the latter is genetic regulated by environmental conditions. Regeneration is a form of self-reproduction and/or adaptation that changes in ontogeny and phylogeny with changes in development or environmental conditions. We can thus alter regenerative ability in the desired direction. Loss of ability to reparative regeneration follows the decreased capacity to destruction and dedifferentiation of basic tissues involved, hence

intensification of these reconstruction phenomena will recover regenerative ability.

The author crystallizes regeneration concepts on organs and tissues and modifies the current view that human CNS lesions are irreversible and that axons do not regenerate after severance. Actually, there is a great similarity between the repair process in the central and peripheral nervous systems although the results differ greatly. We can bridge the gap between intraspinal nerve fibers with solutions of pyrogenic bacterial polysaccharides, DOCA and ACTH but not cortisone, to promote outgrowth of axons across a CNS lesion. We will in time create and maintain a milieu in CNS tissue favorable to the forward growth of axons in the brain and spinal cord. However, we will then be faced with an even greater difficulty than that encountered in peripheral nerves, that of encouraging growing fibers to connect with appropriate endings, so that useful function results.

Two centuries ago Spallanzani laid the foundation for the subsequent research on regeneration. If there were no regeneration there could be no life; if everything regenerated there would be no death. All organisms exist between these two extremes, with a trend toward the latter or else it would be incompatible with reproduction. Every organ appears to have latent power of regrowth within its individual cells. Breakthroughs in regeneration would mean far more than the regrowth of an amputated arm, for a damaged internal organ such as the heart, kidney, or lung might be regrown from a single cell in the laboratory and then transplanted into the same person from whom the tiny cell was taken.

"Man is a born child, his power is the power of growth."

I. NEWTON KUGELMASS, M.D., PH.D., SC.D., *Editor*

PREFACE

This book was written for American physicians, chemists and biologists. I want to present in condensed form the facts about regeneration of organs and tissues, which may not be known to the Western readers. In Western laboratories this problem was studied mainly with amphibia, hydroids, planaria and other animals, possessing high regeneration ability. The results obtained with these animals are presented in a number of excellent monographs such as those of Hay (1966), Goss (1968) or Schmidt (1968). In this book I present an outline of experimental work about the loss and recovery of regeneration ability of organs and tissues in animals, particularly those which lost the regeneration ability in their ontogenesis. Mammals, for example, were among the objects of our studies. The author was engaged in these studies for very many years and his main results were summarized in the book, *The Loss and Restoration of the Regenerative Capacity in the Organs and Tissues of Animals,* Publishing House "Science," Moscow, 1968.

Due to space limitations I shall not dwell upon the results presented in other books dealing with regeneration or related fields. For example, I will not discuss the biochemical data, or the relevance of the well-known chain of information transfer DNA-RNA-protein. To make the presentation more logical, certain unimportant data of the author or of other workers are omitted, the methods are also not discussed. For brevity some approaches to the problems are not mentioned, as well as certain other interpretations of the results obtained. All this compelled me to present the material from the viewpoint that appears to me most probable. This is why the presentation in some places may appear declaratory.

The author recognizes that very little is known in this field, only the ways for future research are suggested. Much remains to be

done. Nevertheless in some chapters, particularly concerning myocard and nerve tissue regeneration, the author found it permissible to make several suggestions about possible practical consequences of the research. Our insufficient knowledge of the problem cannot only excuse certain disputable conclusions of the author, but also can be a stimulus for other investigators.

My most cordial and sincere thanks are due to Professors S. M. Rose, M. Singer, O. E. Schotté, W. Kirsche, J. Altman, M. Mizell, N. P. Sinitsyn, I. V. Torskaya and to Doctors A. Scharf, L. M. Grigoriev, V. P. Kudokotsev, S. Ya. Tuchkova, N. A. Tepliz, K. Yu. Reznikov and E. V. Gusareva, who kindly permitted the use of illustrations from their papers in the present book.

L. V. Polezhaev

Moscow

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ORGAN REGENERATION IN ANIMALS

I

FORMULATION OF THE PROBLEM

PREAMBLE

The regeneration of organs and tissues in different species of animals and wound healing in mammals was extensively discussed in a number of reviews and monographs (Morgan, 1901; Marchand, 1901; Weissman, 1902; Przibram, 1909; Goldzieher and Makai, 1913; Schimkevich, 1923; Korschelt, 1927; Astrachan, 1929; Weiss, 1930; 1939; Zawadovsky, 1931; Philipchenko, 1932; Abeloos, 1932; Nassonov, 1941; J. Needham, 1942; Polezhaev, 1945; 1947, 1947a, 1948, 1968; Polezhaev, Akhabadze, Muzlaeva and Javich, 1965; Voronzova, 1949, 1953; A. Needham, 1952; 1960; Voronzova and Liozner, 1955, 1957; Studitsky, 1948, 1954, 1959; Liozner, 1960, 1962; Rose, 1964; Kiortsis and Trampusch, 1965; Hay, 1966; Schmidt, 1966, 1968; Goss, 1968).

In this small book we shall discuss in condensed form only one problem, the problem of loss and recovery of the ability to regenerate organs and tissues in animals. This problem is one of the most interesting and at the same time most difficult problem in the field of regeneration. And the difficulty of this problem is largely responsible for our insufficient understanding in this field.

It is conventional to divide all animals into two large groups: those which are capable of organ regeneration and those which are not. For example, in fresh water hydra the whole animal can regenerate from a small part. In planaria and rainworms the whole animal can regenerate from a small part. In crayfish and crab the lost claws are regenerated; in newts and axolotles the tails, limbs, jaws and other organs can be regenerated. In contrast, in rats, cats, dogs and other mammals the limbs, tails and other organs are not regenerated and the same is true for man. It should be noted in this connection that in the animals belonging to the first group, not all organs are capable of regeneration, and in the animals of the sec-

ond group some organs still can regenerate. In hydra, for example, the stem usually does not regenerate, while in mammals, including man, tubular bones usually are healed (regenerated) after the fracture. It should be particularly noted that in our discussion we refer to *reparative* regeneration, resulting from the injury or the loss of the organ. We shall not be concerned with the physiological regeneration, where loss and regeneration of organs take place in regular and continuous fashion. Physiological regeneration such as desquamation and repair formation of epithelium are observed in all animals. The specific pattern and intensity of this process, however, are different for different tissues (see Leblond, 1959). Nevertheless the division of animals into two classes by the criterium of regeneration is possible. The problem is, why certain animals are capable of regenerating their organs, while others are not. This calls for the development of methods for stimulation (induction) of regeneration of those organs, which are unable to regenerate under normal conditions.

The discovery of a third group of animals, intermediate between the first and the second, was very essential for our conceptual progress in this field. This group is represented by tailless amphibia (*Anura*) such as frogs, toads and fire-bellied toads. In these animals the ability to regenerate limbs and some other organs exists up to a certain stage in their development, and is abruptly lost at the next one. This loss of regeneration ability occurs in a very rapid fashion. For example, in tadpoles of a grass frog *Rana temporaria,* undergoing metamorphosis, it occurs within two to three days. Thus, animals from this group are both interesting and convenient for studies of factors responsible for the loss of ability to regenerate and for the development of methods for recovery of organ regeneration ability. It will be demonstrated in the following sections that some important principles were established in experiments with animals belonging to this group.

Later it was shown that the concept, postulating the existence of three groups of animals with different ability to regenerate should be revised. It is more correct to say, that there is one sequence of animals, but their ability to regenerate organs, limbs for example, changes during their ontogenic development. In some species, newts or ambystoms, for example, this ability is retained during all their

life, in others, such as frogs, it is lost at a certain stage of their metamorphosis, in still others, as in birds, it is lost at very early stages of their ontogenic development. This loss of ability to regenerate in ontogenesis corresponds to the evolutionary position of these animals in the row of increasing complexity of organization.

Besides tailless amphibia, some other intermediate groups were found. In lizards, for example, the tail regenerates quite well, while the limbs do not.

Further experiments conducted with vertebrates have demonstrated that the lost ability to regenerate limbs could be recovered to some extent in frogs, lizards and even mammals. This will be described in more detail below. The regularities, determining the loss and recovery of the ability to regenerate limbs in vertebrates were determined in a number of investigations. This permitted the beginning of studies on the loss and recovery of the regeneration ability of other mammalian organs. In this connection such organs were studied as the bones of skull vault, tooth tissues, heart muscle, brain and spinal cord which cannot regenerate under normal conditions. The studies were also performed with organs and tissues (axolotl limbs) whose ability to regenerate was suppressed by x-ray irradiation. Investigations, performed with these organs and tissues permitted the establishment of some factors, responsible for the loss and recovery of the ability to regeneration in animals and disclosed some new phenomena, which were not known before. This will be discussed in more detail below.

Before we start the analysis of all this experimental material we have to define some basic concepts relating to regeneration, which will be essential in further discussion. It should be stressed that revision of some older views and introduction of new concepts was a vital necessity.

MAIN CONCEPTS AND PRINCIPLES OF REGENERATION

What do we mean by regeneration? As many other authors we held the view that regeneration represents recovery of impaired or lost part of the animal body (organ, tissue, cell, or part of the cell). This can be a limb in newt or tail in a lizard. The recovered part may have typical or atypical structure—this will be typical or atypical regeneration respectively. Regeneration should not be confused with some other phenomena of recovery, when the lost part

is not regained: these are scarring and hypertrophy. In scarring the impaired region is substituted by a scar of connective tissue and not by specific tissues. In case of hypertrophy, for example, after removal of part of the liver, the wound is smoothly healed and organ residue enters the phase of compensatory growth. This is due to the increase of individual cells (hypertrophy) or their structural elements and to cell proliferation (hyperplasia). Regeneration, hypertrophy and scarring are all very important phenomena, but they follow different laws. Therefore they should not be confused, since this would make the principles underlying regeneration in the strict sense of the word, less clear. Since some authors have a tendency to identify the hypertrophy and regeneration, this must be particularly emphasized.

The regeneration should also not be confused with the regenerative ability. Regeneration is a process, while the ability represents a physiological state of the organ, determining the possibility of regeneration. It may depend on a number of inward or environmental factors. The ability to regenerate is determined genetically and is regulated by conditions of the environment. The ability of newts and inability of rats to regenerate removed limbs is determined by genetic factors. On the other hand the ability to regenerate can always be modified by environmental conditions. We can thus suggest that it is not impossible to try to revive the ability to regenerate which was lost.

We think that the cause of reparative regeneration is the injury (trauma) of tissues.

The basis of regeneration is metabolism, dependent on the cell molecular structure.

As regards the question, whether regeneration can be considered as the primary intrinsic property of an organism or as an adaptation, we can quote Darwin, who wrote: ". . . we may conclude that the several forms of gemmation, and of fissiparous generation, the repair of injuries, the maintenance of each part in its proper state, and the growth or progressive development of the whole structure of the embryo are all essentially the results of one and the same great power." On the other hand, "No doubt the power of reparation, though not always quite perfect, is an admirable provision, ready for various emergencies, even for those which occur only

at long intervals of time." We think that regeneration is a primary property of the living matter, one of the forms of its self-reproduction. On the other hand it can be regarded as an adaptation. This is clear, since regeneration is dependent on the metabolism which is a primary property of living matter but can be considered as an adaptation as well. It can thus be said that regeneration is one of the adaptive forms of living matter self-reproduction. Other forms of self-reproduction include sexual and asexual propagation, division, budding, spore formation, growth, hypertrophy, hyperplasia, and neoplasia both malignant and benign. All of them are characterized by self-reproductive processes involved. Nevertheless each of them has a degree of specificity and differs in this respect from others.

It can be logically concluded that in order to achieve directed alteration of the ability to regenerate the metabolism of the organism or of a given organ must be properly changed, for example, metabolism of the limb in a frog or rat (see Chap. II). But, this cannot be realized by purely empirical approach, just as a necessary number in a telephone book cannot be found by guess. The knowledge of certain basic principles underlying regeneration is important. Three such rules or principles were known for more than a hundred years. These principles are the following: a) The ability of reparative regeneration in animals decreases with the increase in complexity of organization (Darwin, 1868); b) the ability of reparative regeneration decreases with the age of the animal, or with the increasing differentiation or specialization of their organs and tissues (Darwin, 1868); c) during regeneration each tissue produces a similar one (Kölliker, 1865; Waldeyer, 1865).

Even Darwin noted that these rules had exceptions and at present the number of such exceptions is very large. It is known, for example, that in urodeles organs regenerate better than in fishes, which have lower organization. Parts of adult ascidia regenerate well, while in their larvae regeneration is poor. In the adult newt the lens regenerates from the rim of the iris (that is from nerve tissue) and not from epidermis. This is the so-called Wolffian lens regeneration. However exceptions do not make the principles less important. Nevertheless it is highly desirable to find more all-inclusive principles, which would embrace both older principles and

exceptions from them. The classical principles do not contain information relevant to the main problem: what should be done in order to recover the lost ability to organ regeneration. Once and again this leads to the necessity of the search for new principles, which would permit us to attack the problem. On the basis of our own experiments and the data available in the literature, we made an attempt to formulate some new principles governing the phenomena of regeneration (see Polezhaev, 1968). Some of them will be briefly mentioned here.

We came to the conclusion that all the principles of regeneration, as well as known exceptions to these principles, can be explained on the basis of Severtzov's concepts about main directions of evolution (Severtzov, 1934). Severtzov postulated three directions of adaptive evolution: a) Progress—that is increase in complexity of organization; b) regression—simplification of organization; and c) idioadaptation, or evolution in different directions but without change of complexity at a certain level. Moreover, it is considered that evolution is always adaptive and its direction is determined by environmental conditions. We believe that the ability to regenerate always evolved under the action of environmental conditions. The decrease of ability to regenerate with the increase in organization complexity can be regarded as a consequence of progressive evolution. The exceptions are explained by regressive evolution and idioadaptations. The transition from aquatic environment to the terrestrial life (axolotl-frog-rat) resulted in the increase of organization complexity and accompanying decrease of ability to regeneration. When regression took place, for example, in the transition of free swimming larva of ascidia to sedentary life, the organization became simpler and regeneration ability increased. Under certain conditions tissue originated from a similar one, for example, epithelium from epithelium, under other conditions it originated from a different tissue as with the Wolffian lens regeneration. All this can be condensed in one principle which we called the *main principle (law) of regeneration*: the ability to reparative regeneration changes in ontogeny and phylogeny in experimental or pathological conditions is determined by changes of environmental conditions or development. This principal embraces all three older principles and all the known exceptions. On

the other hand, this principle contains an inherent guide to practical work, since by changing environmental conditions one can change the ability of regeneration in the desired direction.

Analysis of biochemical facts available in the literature and their comparison with morphological information permitted us to formulate the *principle of physiological stages* in regeneration (Polezhaev, 1947, 1968). Several other authors were very close to its discovery (Needham, 1952, 1960; Urbani, 1965; Schmidt, 1968). According to this principle any reparative regeneration must contain at least two physiological stages, with a different type of metabolism. This different metabolism serves as a basis for the onset of morphological changes. These stages are the following: A) stage of destruction and dedifferentiation, which is characterized by proteolysis, glycolysis, lowering of nucleic acid concentration and of pH in the tissues. Morphologically this stage is characterized by accumulation of histologically nondifferentiated regenerative material in the lesion zone. The second stage is a stage of growth and differentiation, characterized by prevalence of protein synthetic processes over the proteolysis, return to aerobic respiration, increase of nucleic acid concentration and increase of pH to the normal values. From the morphological standpoint this phase is characterized by growth and differentiation of regenerative material in the lesion zone. The third stage of growth is not sufficiently characterized from a biochemical viewpoint. A. Needham (1952, 1960) in a similar analysis mentioned two first stages, which he calls regression and progression stages respectively.

These physiological phases have different expression during the regeneration of homologous organs in animals of different species and of different age. Our experiments have demonstrated (Polezhaev, 1968) that the phase of destruction and dedifferentiation is clearly expressed in animals with high ability to regeneration and is not obvious in animals with low regeneration ability. If the destruction and dedifferentiation stage is augmented by some means, for example, by stimulation of proteolysis or nucleic acid degradation the second stage of regeneration can be intensified as well. This may lead to partial or complete recovery of the ability to regenerate. The destruction of tissues is accompanied by the increase of nucleic acid and protein synthesis, while degeneration and

degradation of tissues lead to its decrease.

Another important principle of regeneration is the *principle of organo-specificity* or the regional principle. It permits understanding of what should be done for selective physiological influence on the regeneration of a certain organ, for example, an inner organ.

It is known from experimental embryology that anlages of different organs in development have different morphogenetic abilities and different inducing properties (Spemann, 1936; Filatov, 1939; Holtfreter, 1933 etc.). The body of the adult animal consists of a number of territories, and each of them shows a particular specific ability to regeneration (Guyénot et Schotté, 1927). Each organ has different biochemical and immunological specificity (Abderhalden, 1921; see Vyasov, 1963). The anlage can be affected (stimulated or retarded) by homogenates, extracts or lysates from homologous organs. These preparations can be administered to the recipient organism by different means (Murphy, 1916; Dantschakoff, 1918; Weiss, 1947; Ebert, 1954; Rose, 1955 etc.). Homogenates or transplants of certain organs when labelled by colloidal stains or radioactive isotopes are absorbed by similar or homologous organs (Belonovsky et Miller, 1926; Ebert, 1954; Mahler, Walter and Bulbenko, 1956). In this way limb, skeleton or muscle regeneration can be stimulated in fire-bellied toads or frogs (Polezhaev and Ramenskaya, 1950; Tumanishvili, 1958, 1965; Kudokotsev, 1960 etc.). Thus, under certain circumstances, substances prepared from a given organ can affect its regeneration. The method of preparation of these substances, their dosage, method of administration, etc. must be determined experimentally.

Results of our extensive experimental work, which will be presented below, enabled us to make one more important generalization: *the loss of ability to reparative regeneration in ontogeny and/or phylogeny of animals is due to the decreased ability to destruction and dedifferentiation of basic tissues comprising these organs. Intensification of these phenomena is necessary for the recovery of regeneration ability.* Probably tissue destruction liberates certain biologically active substances possibly of protein or nucleic acid nature, which lead to tissue dedifferentiation, activate cell proliferation ability and finally result in tissue or organ regeneration. It is possible that these agents stimulate protein or nucleic acid synthesis.

It should be noted that we define differentiation as the increase of complexity and specialization of cell, tissue or the organ. In contrast dedifferentiation represents the simplification, the approach to the embryonic state. Morphogenetic properties of the dedifferentiated tissue need not be changed. The literature does not give clear answer to the question whether dedifferentiated tissues become pluripotent or totipotent.

Thus we formulate our task. How can we change the course of events in such way, as to recover the ability to regenerate organs and tissues in animals that do not possess it? How can this be applied to human beings?

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II

RECOVERY OF THE LOST ABILITY TO REGENERATE LIMBS IN VERTEBRATES

THE SURVEY OF LITERATURE

The attempts to recover the lost ability to regenerate organs in some animal species and in man have a long history, but they were largely unsuccessful. For example Morgan (1908) assumed that the loss of ability to regenerate limbs in adult frogs was due to enzymic alterations in tissues. In order to modify the enzymic properties and to recover regeneration ability he transplanted tissue from tails and limbs of tadpoles able to regeneration onto the wound surfaces of such frogs. The regeneration was not observed. In order to evoke regeneration of the amputated fibular bone in human infant, Bier (1923) transplanted fragmented bone of a dog in its place, but this brought no success. The bone did not regenerate. Weiss (1930) transplanted young (undetermined) or developed (determined) tail regeneration blasthems to the wound surfaces after limb removal in lizards. In the first case blasthems did not develop, in the second tails were formed. The author suggested that the inability to regeneration was due to disappearance of the "morphogenetic field." Nothing is known about the nature of this field and about possible ways of its change necessary for the recovery of regeneration. Ide-Rozas (1936) came to the conclusion that the inability to regenerate extremities was due to the inability of cells to proliferate but this was not proved experimentally.

Limb regeneration in tadpoles of different species has been known for a very long time. It was observed by a number of authors (Spallanzani, 1769; Barfurth, 1895; Kochs, 1897; Byrnes, 1904; Kammerer, 1905; Bauer, 1905; Marcucci, 1916, and others). These results lead to the firm conclusion that regeneration of forelimbs and hind limbs and tails in frog, toad and fire-bellied toad

tadpoles is very efficient up to a certain stage in their development. Later this ability is lost. In *Xenopus laevis* tadpoles the ability for a typical regeneration survives even after metamorphosis (Skowron and Komala, 1957; Dent, 1962). There are tables and special nomenclature for designation of different metamorphosis stages in tadpoles (Blacher, 1928, etc). It was established that ability to undergo regeneration remains longer at distal levels of limbs than at proximal ones (Polezhaev, 1939; Schotté and Harland, 1943) and this might be due to differences in the degree of skeleton differentiation (Polezhaev, 1948). If frogs fore limbs are removed distally (in the wrist), limited and atypical regeneration is observed (Polezhaev, 1947, 1948; Thornton and Shields, 1945).

Systematic search for factors responsible for the loss of limb ability to regeneration was begun by Guyénot (1927). He studied the effect of inner humoral medium on the limb regeneration ability in tailless amphibia. In his experiments he xenoplastically transplanted toad tadpole limbs, which were not capable of regeneration, and tails that were capable of regeneration to salamander larvae, which possessed the regeneration ability. Transplanted extremities did not regenerate, while transplanted tails did so. From these results Guyénot concluded that blood did not contain the substances inhibiting the regeneration, but later this demanded corrections.

Liozner (1931) continued Guyénot investigations. In his experiments he conducted homoplastic transplantation of limbs that were able or unable to regeneration in grass frog tadpoles. The recipient tadpoles at different stages of metamorphosis were used. Limbs of young tadpoles retained the ability to regeneration after the transplantation to older tadpoles, which already lost the regeneration ability. The limbs unable to regeneration did not regenerate when transplanted to the animals capable of regeneration. It was shown only later that tadpoles at the advanced stages of development possessed an inner humoral medium, which inhibited limb regeneration, while humoral medium of young tadpoles facilitated the survival of limb regeneration ability (Borsook, 1935, 1936; Polezhaev, Ginzburg, 1939, 1941). It may well be, that regeneration is also suppressed by a thyroid hormone (Liozner, 1931, 1936).

An interesting fact concerning regeneration was established by

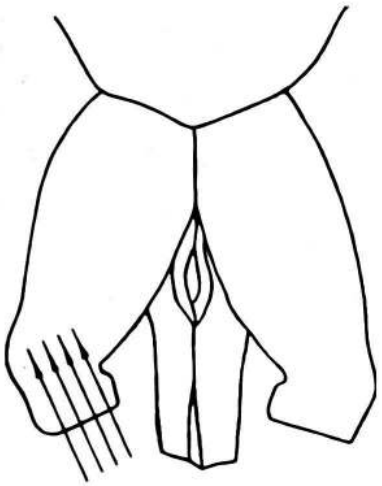
Liozner (1931). He found that transplantation itself stimulated the regeneration, and when transplantation was conducted at the critical stage (stage IIa according to Blacher, 1928) it could result in the reappearance of the lost ability to regenerate organs in tadpoles. Unfortunately these studies were not accompanied by histological analysis.

Further studies on the recovery of the lost ability to regeneration were quite systematic and purposeful. They were characterized by several new approaches and by the establishment of some new important facts described below.

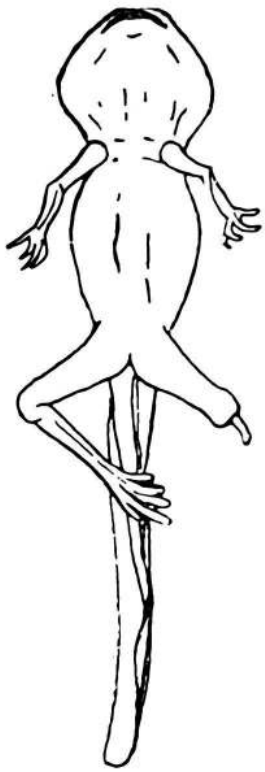
THE RECOVERY OF LOST ABILITY TO REGENERATE LIMBS IN TADPOLES

We turned our attention (Polezhaev, 1933) to the fact that the ability to regenerate extremities in tadpoles is lost very suddenly. When limbs of *R. temporaria* tadpoles are removed at the stage I (Blacher, 1928) the blastema is formed on the wond surface and removed limb is regenerated. When similar operation is conducted at a later stage (stage IIa, Blacher, 1928) the blastema does not form and limb does not regenerate. The absence of blastema formation is probably connected with the absence of cell accumulation in the wound region. We decided to stimulate such accumulation and to effect this, we additionally traumatized the organ residue after the amputation of the extremity. We hoped that the products of tissue destruction, the so-called "wound hormones" (Bier, 1917; Haberlandt, 1922) would stimulate the process of proliferation.

In these experiments both tadpole hind limbs were removed somewhat more distally than the knee. The operation was performed at stages IIa and IIb, when the ability for regeneration was already lost. The left limb was not subjected to any further treatments and served as a control. The right one was additionally traumatized by the needle in distal-proximal direction, about twenty cuts were made (Fig. II-1A). In the control only smooth healing was observed, while the experimental limb showed regeneration. At first these experimental limbs became red and swollen, this was followed by the blastema formation on the wound surface, which grew and developed into a typical five-toed extremity or an atypical one with 4-2 toes (Fig. II-1B,C,D).



1A



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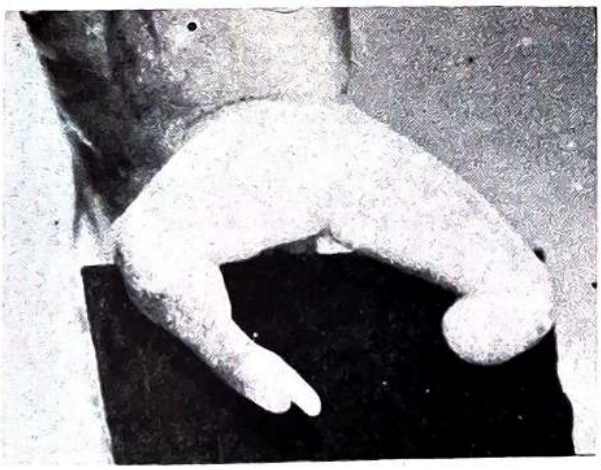

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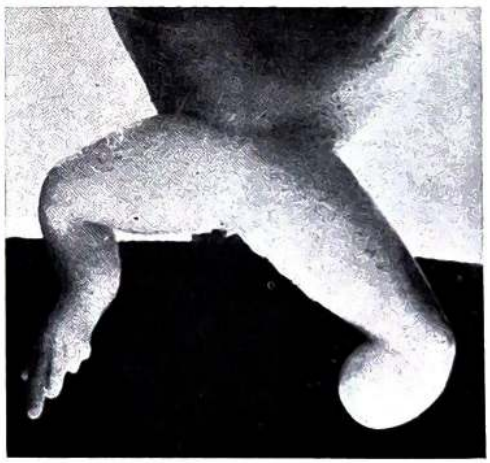
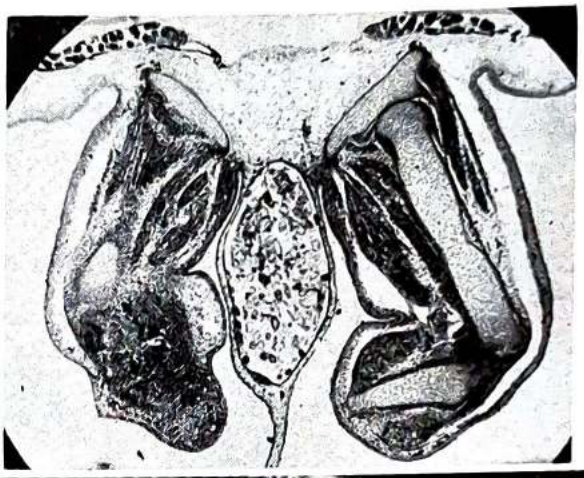
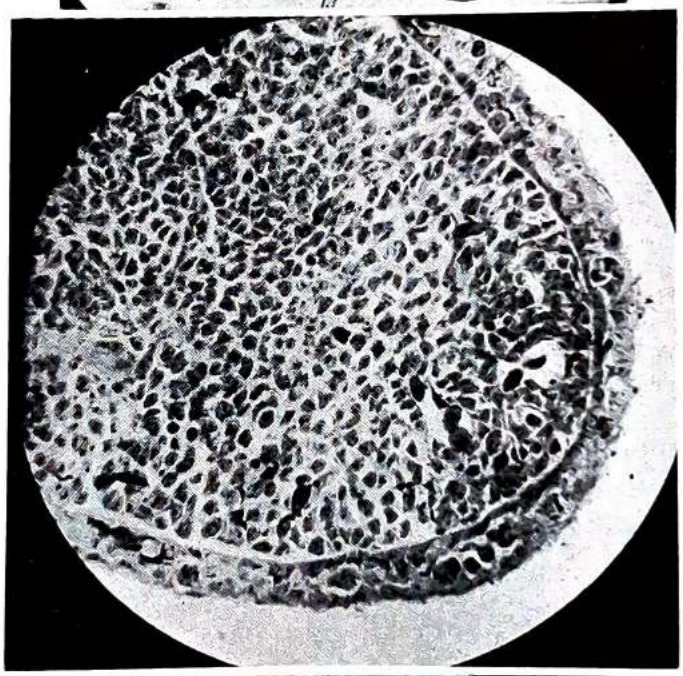


1D

Figure II-1. Recovery of lost regenerative capacity in tadpoles *Rana temporaria*. Top left, Course of operation. Ventral side view. Arrows indicate traumatization of the stump of the right limb; the left limb served as a control. Remaining parts of the figure show the results of the experiment. Complete or partial regeneration of the limb under study and no regeneration of the control limbs.



2A



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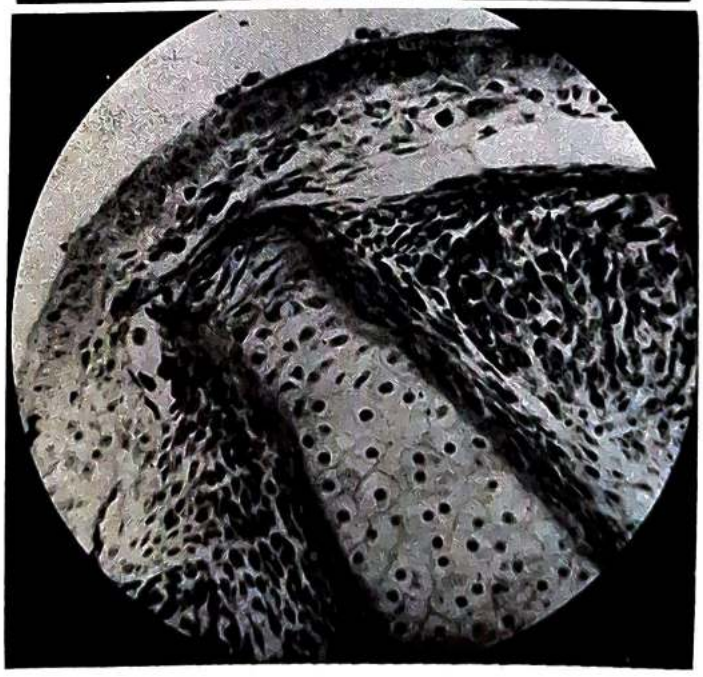

2C

This result confirmed our basic idea that the loss of ability for regeneration was due to insufficient accumulation of cells in the wound region after the amputation of extremities. Microscopic studies demonstrated, however, that traumatization resulted more in the augmenting of destruction and dedifferentiation of the tissues of stump than in the stimulation of proliferative activity. The number of mitotic cells in control and experimental limbs was approximately the same, but the extent of tissue destruction and dedifferentiation was quite different (Polezhaev, 1935, 1936, 1939). In the control limb tissues of mesodermal origin (cartilage, bone, muscle and connective tissue), which will be referred to as mesodermal tissues, showed insignificant destruction, did not dedifferentiate; their wound surface smoothly healed, covering with connective tissue. No regeneration was observed with the control limb. In the experimental limb deep tissue destruction was evident, it was accompanied by dedifferentiation and conversion into a mass of uniform cells, which intensively migrated to the wound surface epithelium forming the regeneration blastema (Fig. II-2). This blastema was growing, differentiating and finally was converted into the regenerated limb.

In several other works we demonstrated that the recovery of regeneration ability of tadpoles extremities could be observed not only at the critical stage IIa (Blacher, 1928) but also at later stages, in this case the regenerations in froglings might be observed

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Figure II-2. Frontal section of limbs of tadpole. Shown six days after operation. Top, General view of limbs. Left, The experiment—destruction and dedifferentiation of tissues in the stump and formation of the regeneration bud after traumatization; Right, Control—no destruction and dedifferentiation of tissues in the stump and uneventful healing of the wound; Middle, Regeneration blastema of the experimental limb; Bottom. No regeneration of the control limb.

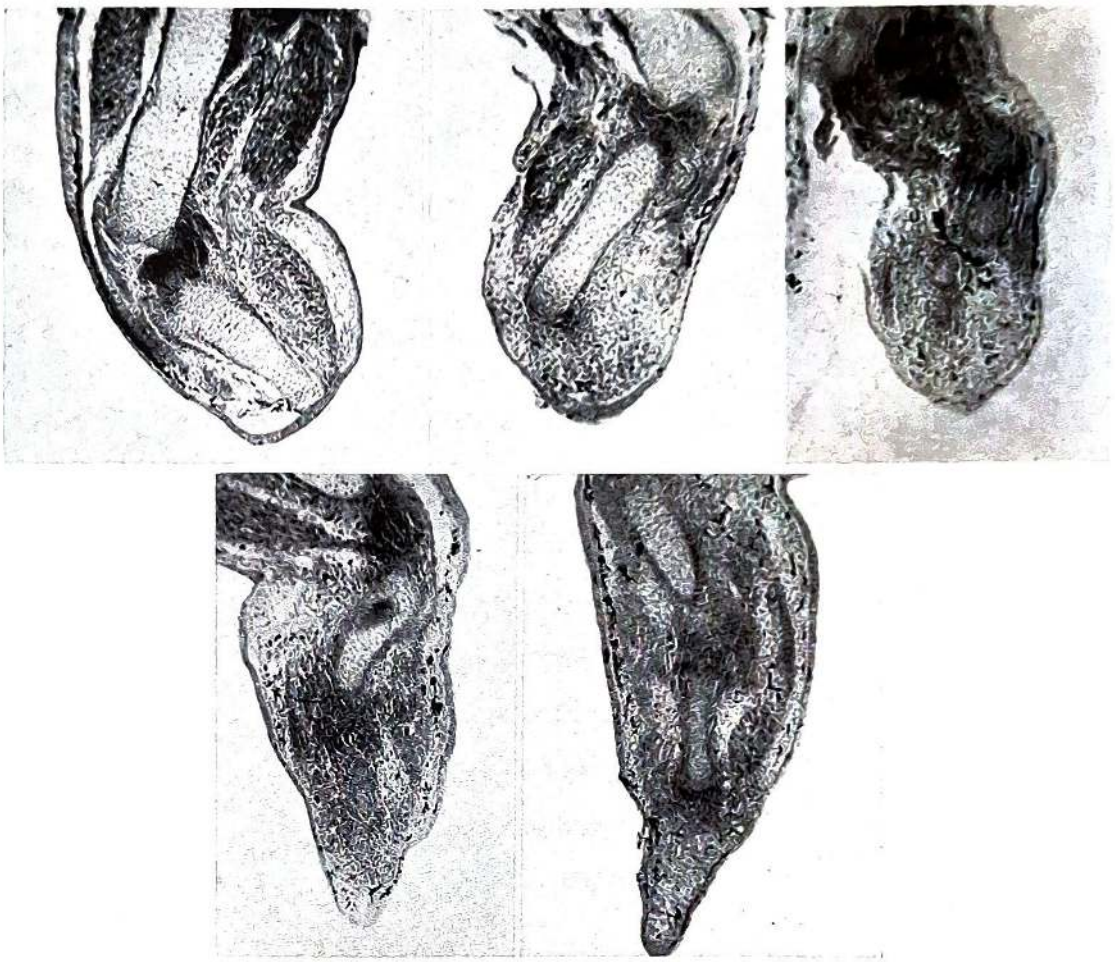


Figure II-3. Regeneration of limbs in tadpoles *Rana temporaria* after amputation at the stage I, when they are capable of regeneration. Top left, After twelve hours; Top middle, After three days; Top right, After four days; Bottom left, After nine days; Bottom right, After eleven days.

(Polezhaev, 1948). It was further shown that after the limb amputation at the stage I, when they regenerate quite well, mesodermal tissues become destroyed and dedifferentiated (Fig. II-3); at later stages no such destruction and dedifferentiation was observed (Fig. II-4A,B) (Polezhaev, 1940, 1946, 1948). These results were confirmed with tadpole regeneration in *R. sylvatica* (Forsyth, 1946). The studies of limb regeneration in different vertebrates at different levels of organization, in particular during the transition from aquatic way of life to the terrestrial one (axolotles-frogs-lizards-mice, rats) have demonstrated that in animals with high ability for regeneration, such as axolotles, early tadpoles and newts amputation lead to destruction and dedifferentiation of the mesodermal tissues of the organ residue. In those animals, which cannot regen-

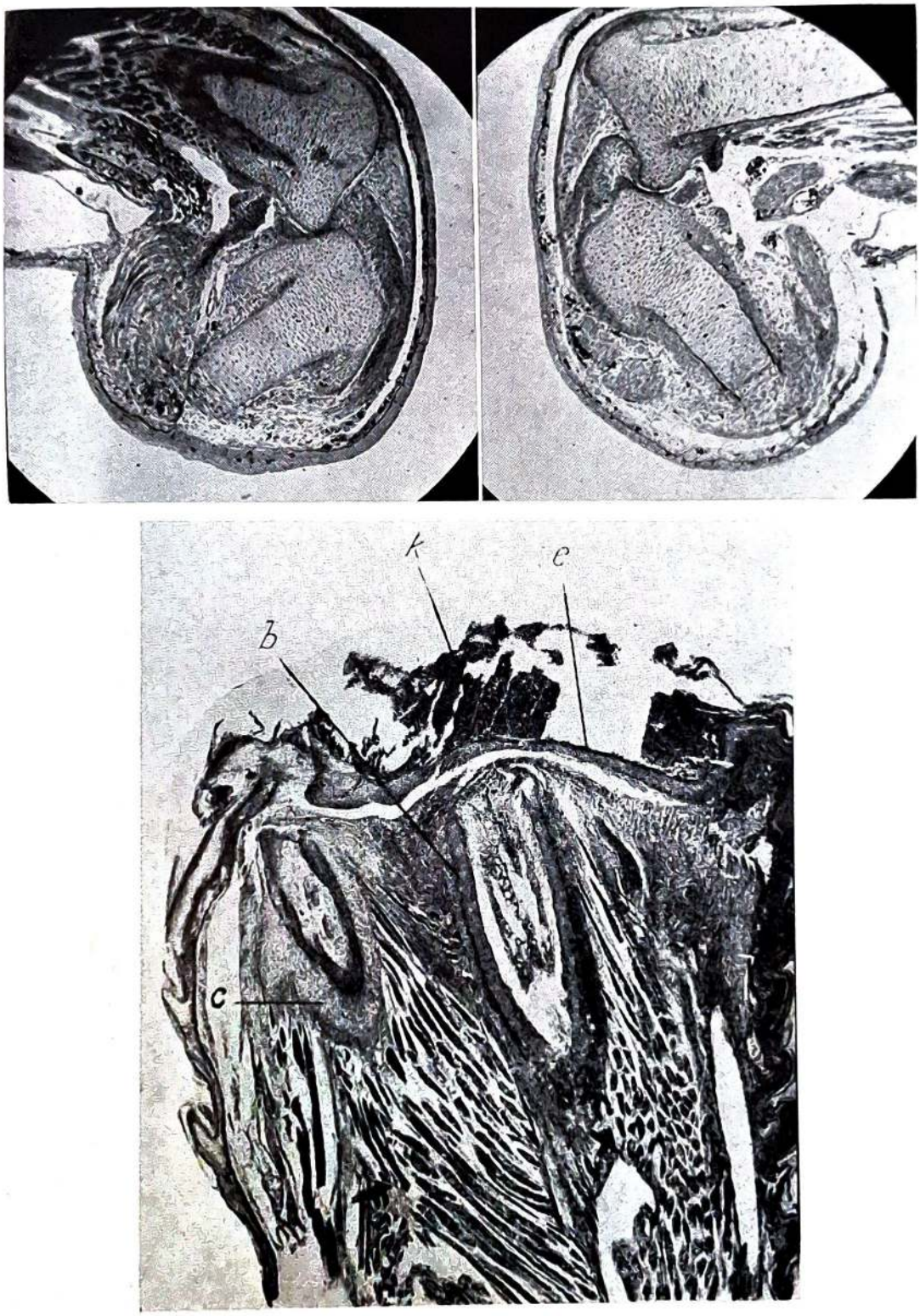


Figure II-4. No regeneration of limbs which lost regenerative capacity. Top left, After three days; Top right, eight days after amputation of hind-limbs in tadpoles *Rana temporaria* at the stage IIb; Bottom, twenty-three days after amputation of forelimbs in adult lizard *Lacerta agilis* (e-epithelium, b-old bone, c-cartilage callus, k-scab).

erate the limbs (tadpoles at late stages of metamorphosis, adult frogs, lizards, mice and rats) mesodermal tissues of the organ residue are not destructed. No dedifferentiation is observed and wound surfaces are smoothly healed (Fig. II-4C) (Polezhaev, 1935, 1939, 1940, 1941, 1948; Barakina, 1951, 1951a, 1952, 1952a). Dedifferentiation of skeleton limbs and connective tissues after limb removal in urodele amphibia was also described by American authors (Butler, 1933, 1935; Thornton, 1938, 1938a, Schotté and Harland, 1943, and others). The absence of dedifferentiation after limb removal in lizards was described by a number of authors (Barber, 1944; Umansky, 1946; Polezhaev, 1948). The same was observed after toe removal in mice (Schotté and Smith, 1959).

RECOVERY OF REGENERATION ABILITY OF LIMBS IN ADULT FROG, LIZARDS AND MAMMALS

Several authors were successful in their attempts to recover the lost ability to regenerate limbs in frogs. Rose (1942, 1944, 1945) obtained the regeneration by treatment of frog wound surfaces

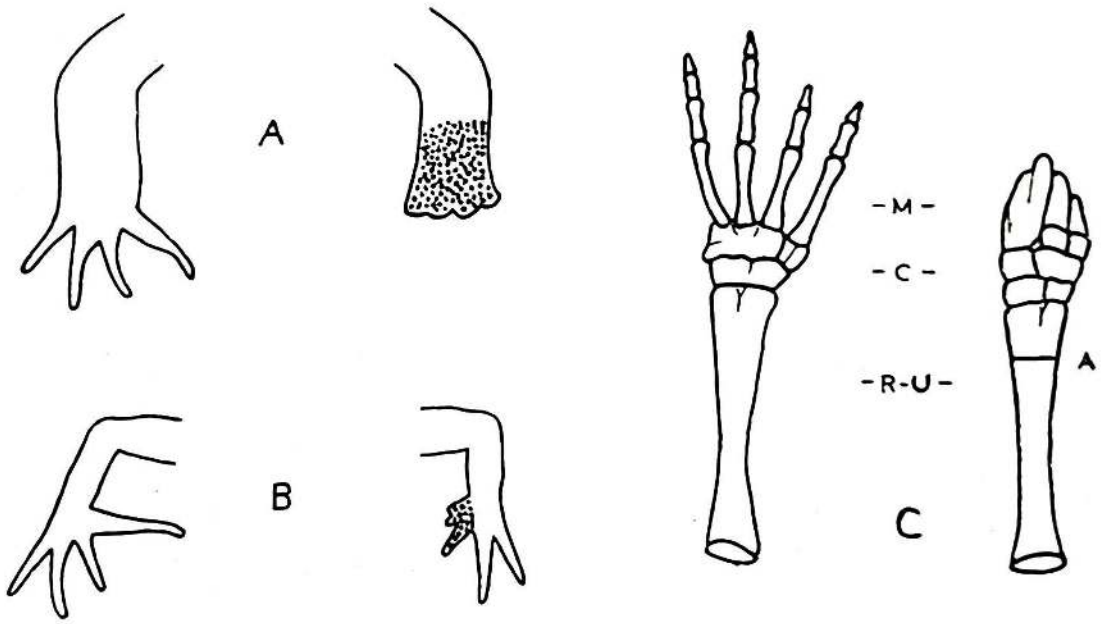


Figure II-5. Comparison of salt-induced regenerants and normal limbs. (Rose, 1942). A, Regenerant stippled. Amputation seven months earlier when frog was a year past metamorphosis. Treated for eight weeks. B, Amputation four months earlier when frog was one month past metamorphosis. Treated for three weeks. C, Skeletons of regenerant and normal arm compared. A, level of amputation. Regenerated skeletal elements—R-U, part of radioulna; C, carpals; M, metacarpals.

with hypertonic sodium chloride solution and by transplantation of young tadpole skin pieces to frog limbs. He worked with such species as *R. clamitans, Rana catesbiana, Rana palustris* and *Rana pipiens* (Fig. II-5). In our experiments with *R. temporaria* and *Bombina bombina* the regeneration resulted from different treatments, for example, wound surfaces were treated with hypertonic

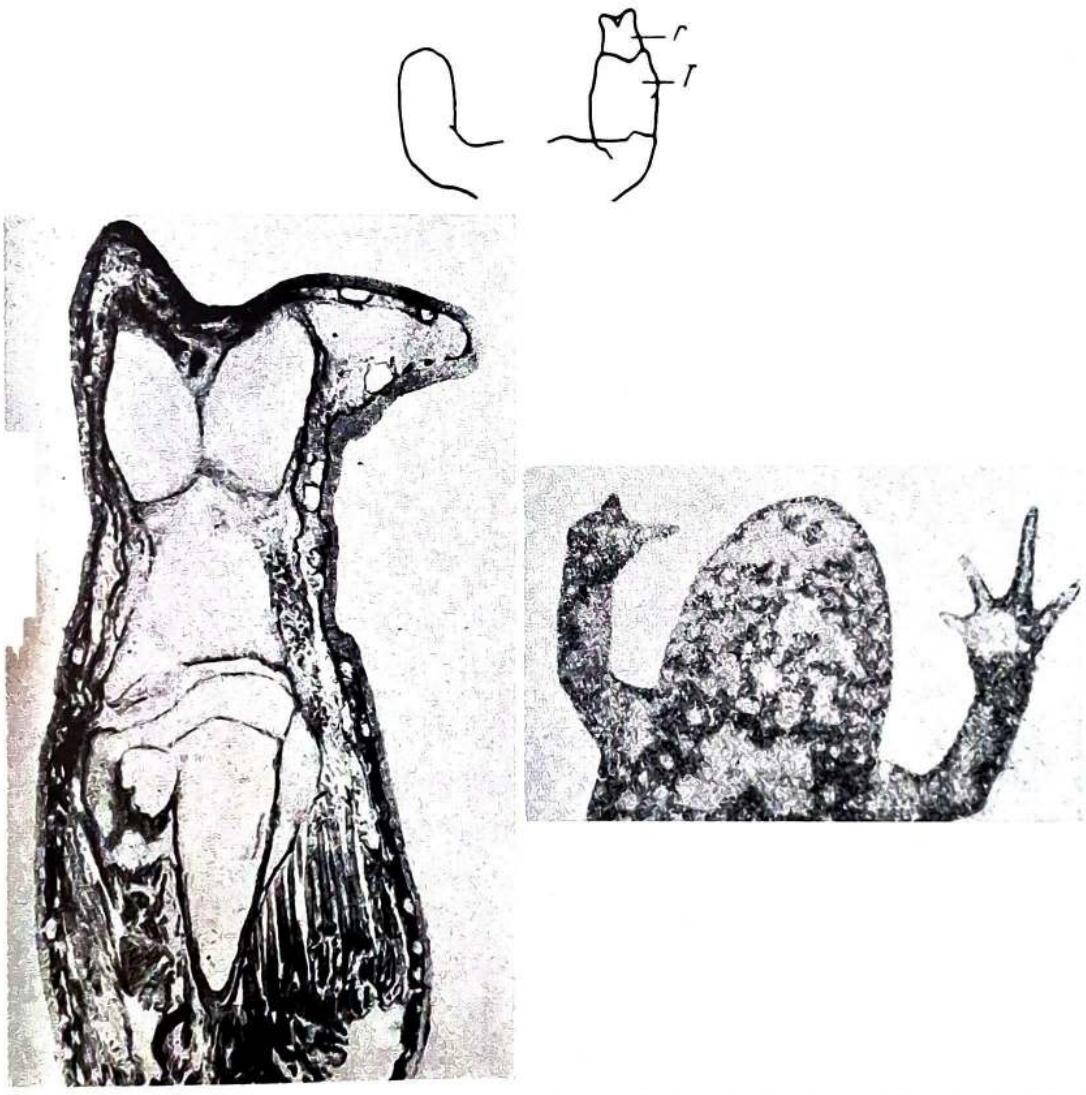


Figure II-6. Induction of regeneration of forelimbs in adult fire-bellied toads. Top left, Limbs in adult toads *Bombina bombina*; Left-control; Right-experiment; r-regenerant; T-skin transplantant preserved according to V.P. Filatov 1945. Right, Histological section of regenerated forelimb in adult toad *Bombina bombina* eight months after amputation in the experiment with tight ligation; r regenerant. Bottom left, Induction of forelimb regeneration (on the left) four months after amputation of the arm in adult fire-bellied toad subjected to double irradiation by near UV-light for forty-five minutes each time (Rogal, 1951a).

sodium chloride solution, 5% lactose solution, 0.8% sodium bicarbonate or 10% iodine solution. Similar effect was observed when conserved skin cuff, kept at +2, +7°, was transplanted or, when tight ligature injuring mesodermal tissue was applied; transplantation of hydrolyzed cartilage lead to the same goal (Fig. II-6) (Polezhaev, 1944-1948). Singer *(1954) induced the regeneration of forelimbs in frogs connecting them with n. ischiadicus innervating the hind limb (Fg. II-7). Similar effect is observed when

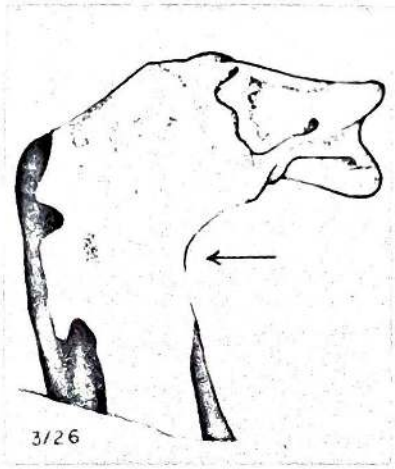


Figure II-7. Forelimb regeneration in adult frog *Rana pipiens*, induced by deviation of the sciatic nerve. An arrow indicates the level of amputation. 5 months after operation (After Singer, 1954).

frog embryonic or larval tissues are implanted into the limbs (Malinin and Deck, 1958; Malinin, 1960) or when limb nerves are irritated with the electric current (Bodemer, 1963; Smith, 1967). Good results were obtained on frogs with adrenals implanted under the skin (Fig. II-8) (Schotté and Wilber, 1958). Similar effects were induced by tissue extract and administration (Kudokotsev, 1960). It has been shown, that various chemical and biological treatments stimulate the destruction and dedifferentiation of tissues. Additional innervation stimulates proliferative processes and accumulation of blastema cells at the limb wound surface.

Injections of parathyroid hormone resulted in distinct softening

*According to the theory of Singer (1965) limb regeneration in vertebrates demands the participation of a certain amount of nervous fibers, whether they are sensory, motoric or sympathetic. According to his concept (Rzehak and Singer, 1966), the loss of ability for regeneration in lizards and mice is due to the decrease of nervous fibers number per square unit in their limbs .

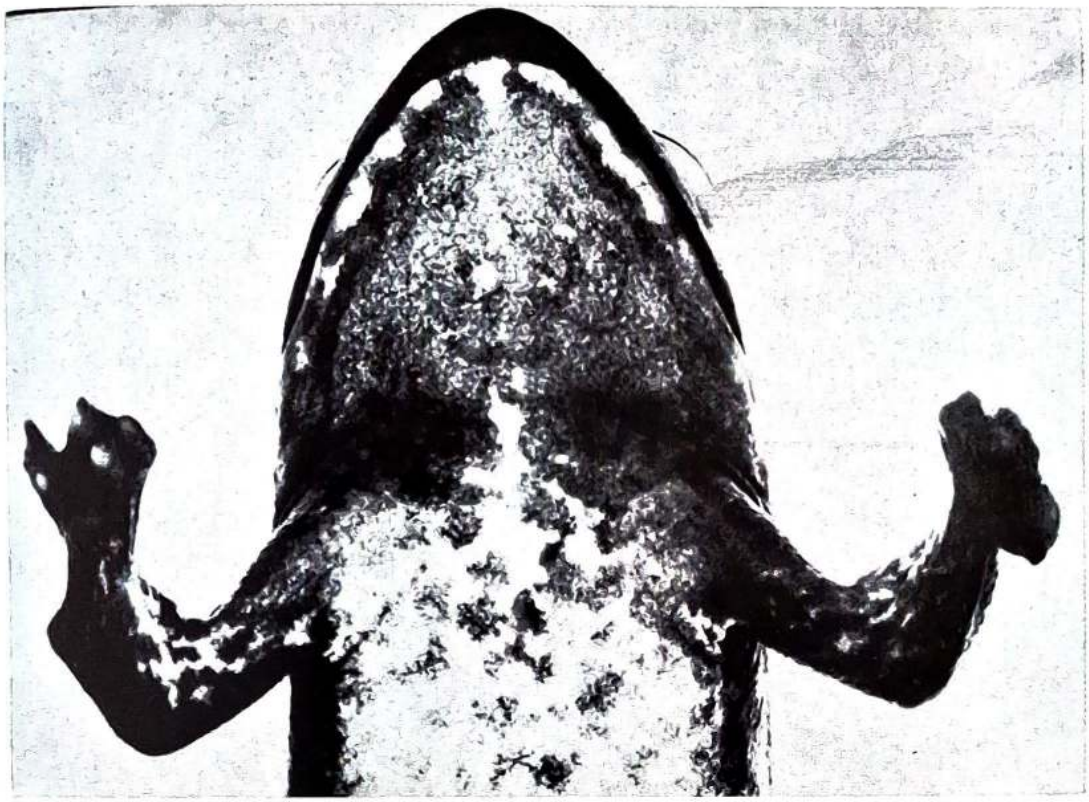


Figure II-8. Regeneration of forelimbs in young frog *Rana clamitans* after grafting a supernumerary suprarenal gland into the mandible (Schotté a. Wilber, 1958).

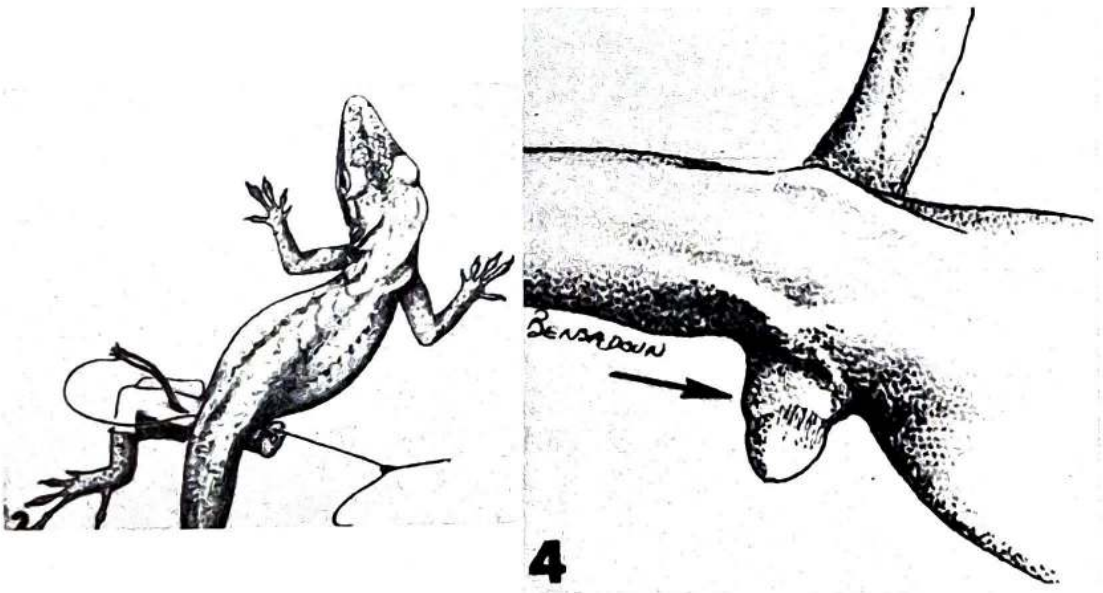


Figure II-9. Induction of hindlimb regeneration in adult lizards, induced by deviation of the nerve (Singer, 1961). Regeneration of atypical limb in lizard *Anolis carolinensis* (Singer, 1961). Left, Course of operation. Right, Results of the experiment.

of the body skeleton leading to the regeneration of atypical limbs in lizards (Umansky and Kudokotsev, 1951). Similar result was obtained when the stump remaining after hind leg removal in lizards was connected with an additional nerve (Fig. II-9, 10) (Singer, 1961; Kudokotsev, 1962, 1963). Rogal (1951) induced D and A avitaminosis in white rats. This type of avitaminosis facilitates the ability of the skeleton to be destroyed. In newborn rats, delivered by such animals, wrist amputation leads to the regeneration of large protruded formations with toe-like structures which even contained the nails (Fig. II-11). Umansky and Kudokotsev (1952) observed the regeneration of stumps containing epiphyses of an elbow and radial bones, when newborn rats used for this experiment received parathyroid hormone before limb removal (Fig. II-12). In our unpublished experiments, conducted as early as in 1949, injection of vitreous body preparations to mice re-

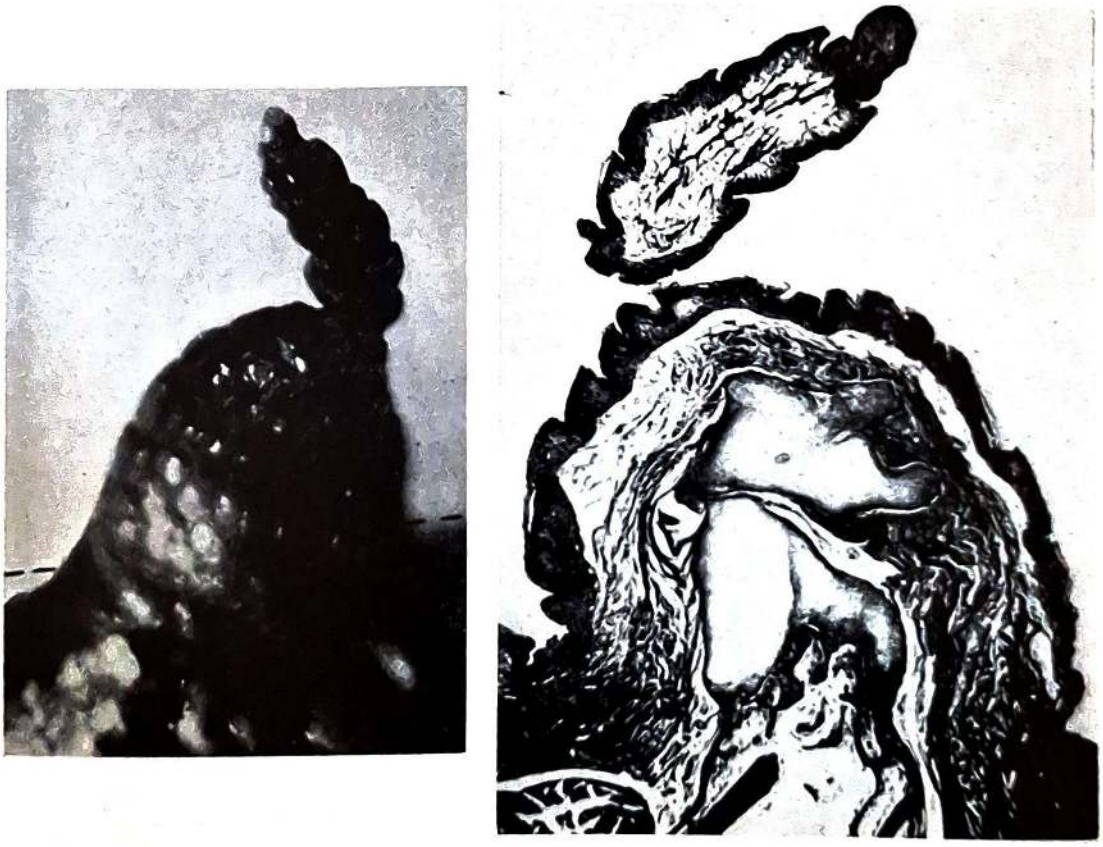


Figure II-10. Limb regenerant in lizard *Lacerta agilis* induced by deviation of the nerve (Kudokotsev, 1962). Left, General view of the regenerant; broken line indicates the regenerant area. Right, Longitudinal section of ninety-days limb regenerant; staining with hematoxylin-eosin (magnification: ocular 4, objective 3.7).

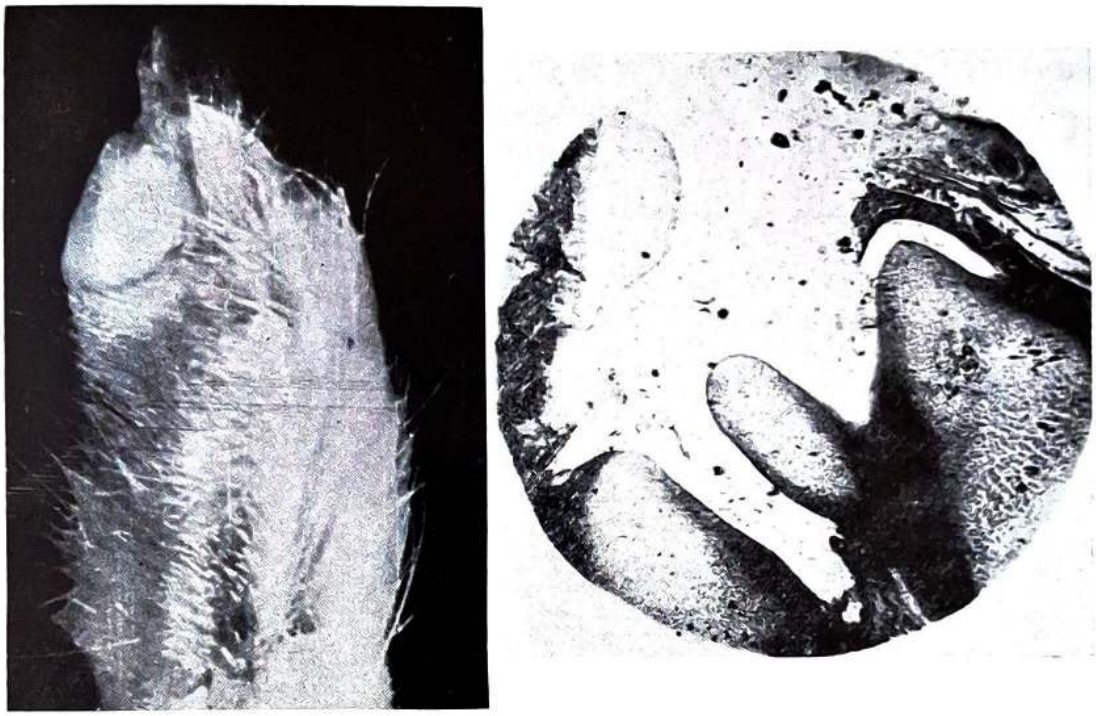


Figure II-11. Induction of limb regeneration in newborn rats. An experiment with D and A avitaminosis (Rogal, 1951). Left, General view of limb regenerant; Right, Cartilages formed in the wound area.

sulted in the formation of finger-like processes at healing stumps. This process was usually found in the neighborhood of the first rudimentary toe. When the first toe was simply removed, similar type of regeneration was observed (Fig. II-13).

Scharf (1961, 1963) in the United States removed two fingers from the fore limb of two-day-old rats and treated the wound by trypsin and by calcium chloride solutions. This led to the regeneration of two short toes which later became covered by nails (Fig. II-14).

Kudokotsev and Kuntsevich (1965) and Kudokotsev (1966) stimulated cartilage regeneration on the wound surfaces of limbs of two to seven day-old rats by injection of cartilage extract; treatment of tail residues after tail removal by trypsin or calcium chloride solutions resulted in the restoration of the amputated part of the vertebra.

Mizell (1968), Mizell, *et al.*, (1970) conducted experiments with newborns of North-American marsupial opossum *Didelphis virginiana*. After the amputation of fore or hind legs, smooth wound healing was noted, however, when brain pieces obtained from frogs or other opossums were implanted into the stump, the re-

generation of atypical hind legs was observed. It is important to note that at this developmental stage limb tissues of opossums are only weakly differentiated, nerve fibers are not covered with myelin layer and thymus is not developed. It means that organism

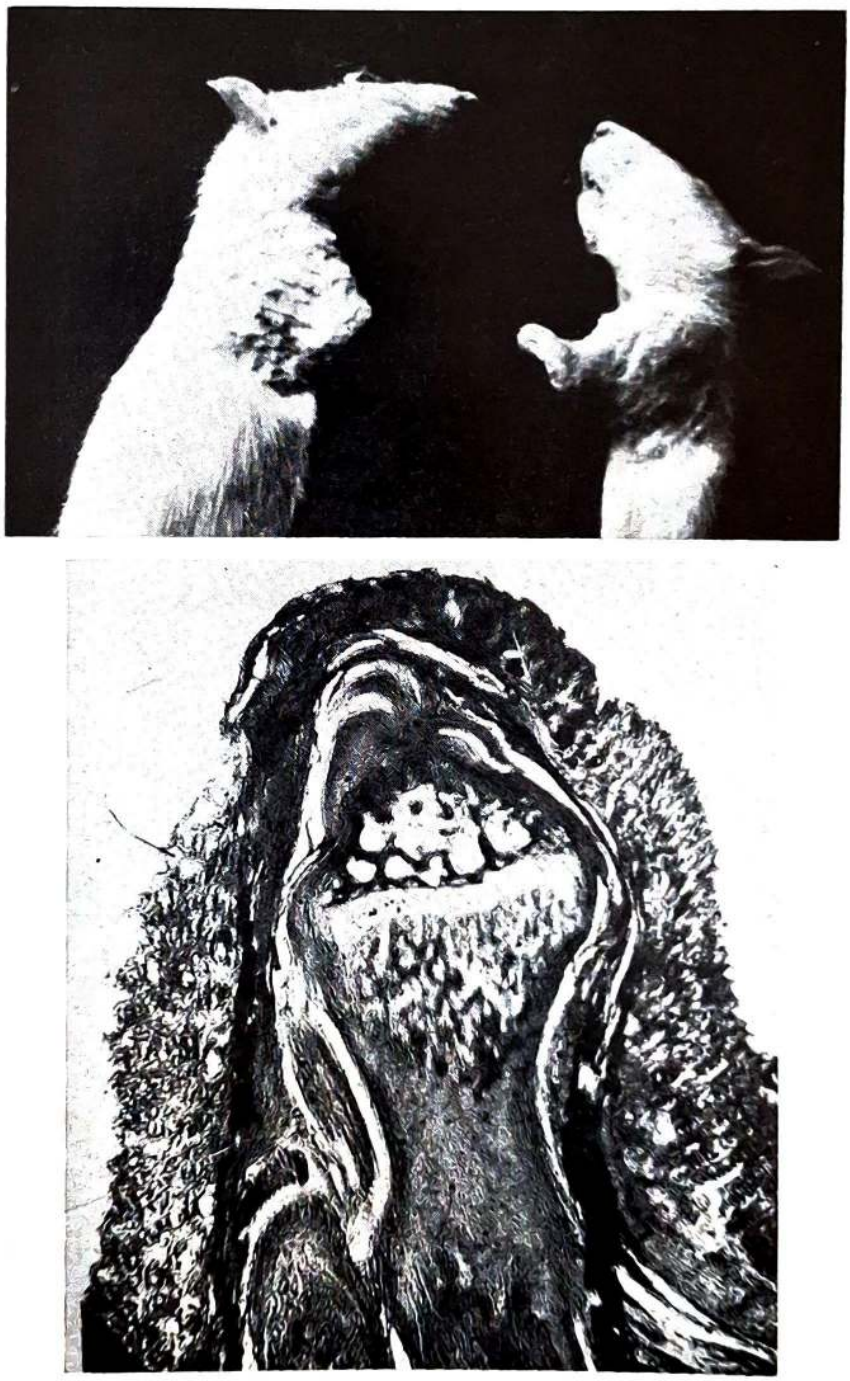
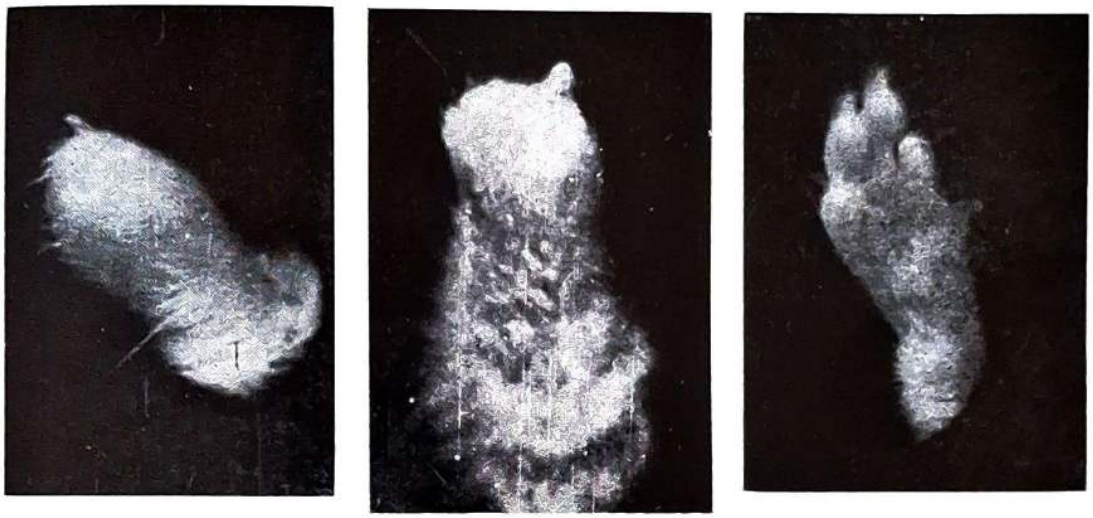


Figure II-12. Results of the experiment on stimulation of limb regeneration in young rats under the use of parathyreocrine (Umanski a. Kudokotsev, 1952). Top, General view; on the left is the control and on the right the experimental animal with limb regenerant. Bottom, Longitudinal section of limb regenerant; staining with hematoxylin-eosin (ocular 4, objective 3,7).

protective systems are not yet functional at this stage. Brain implantation leads to tissue dedifferentiation, blastema formation and



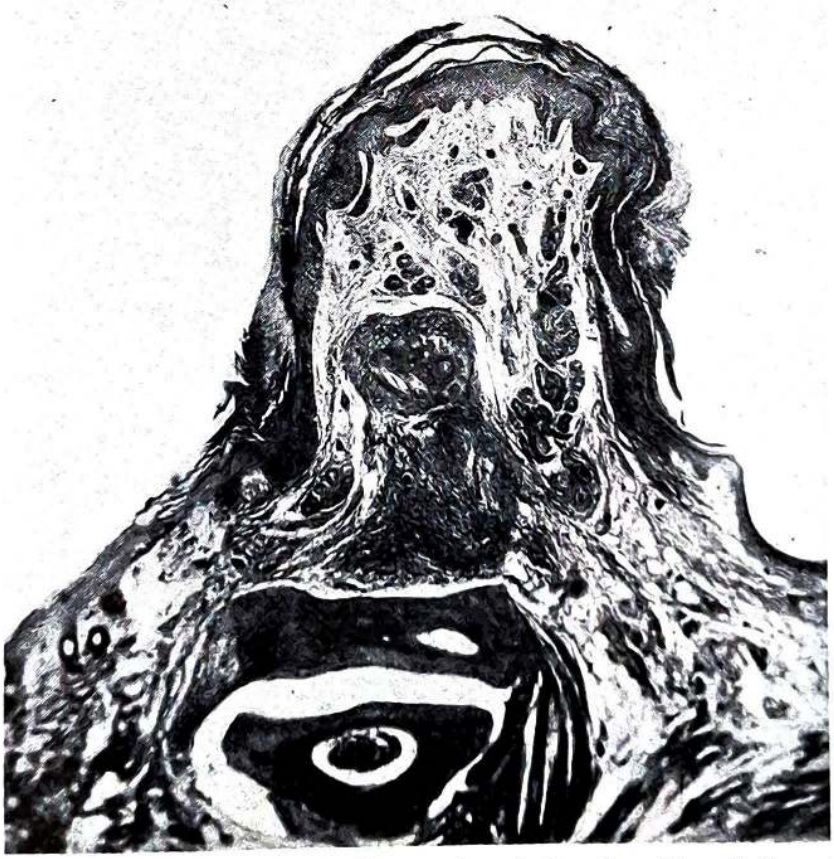


Figure II-13. Regeneration of fingers in adult mice. Top left and middle, Formation of finger-like outgrowths at the base of the first finger by administration of vitreous-body preparation; Top right, Outgrowth formed after amputation (its level is indicated by an arrow) of the first finger; Bottom, Longitudinal histological section of regenerant formed at the level of the first finger after amputation and injection of vitreous-body preparation.

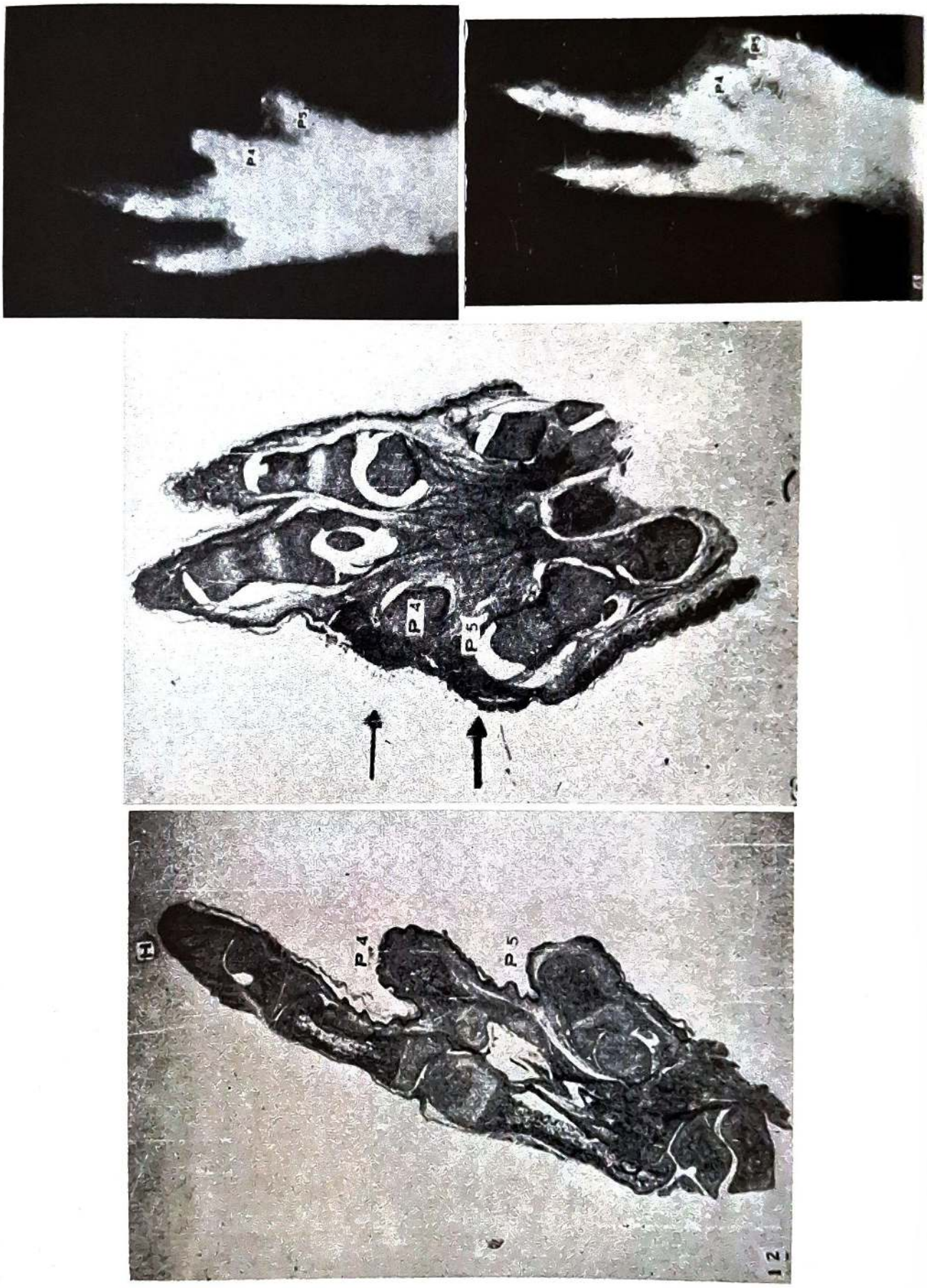


Figure II-14. Induction of regeneration of fingers in newborn rats. An experiment on treatment of wound surface with solutions of trypsin and calcium chloride (Scharf, 1961). Top left and right, Control. Bottom left and right, Experiment.

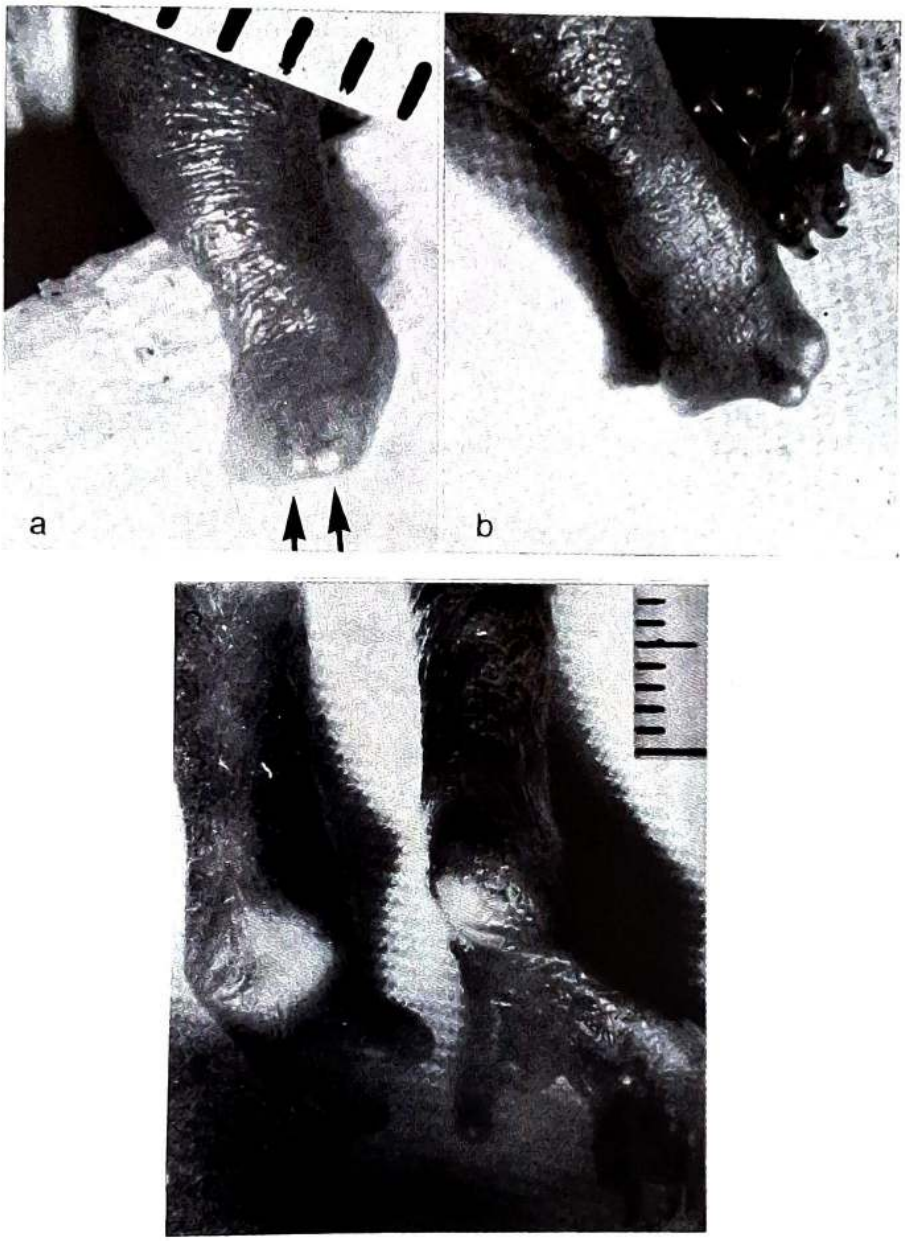


Figure II-15. Induction of regeneration of hindlimbs in newborn opossum *Didelphus virginiana* (Mizell *et al.*, 1970). Left, Hindlimb of twenty-four-day-old opossum. Nervous tissue from young opossums cerebrum was transplanted when two days old; limb was amputated four days later. After eighteen days of regeneration, early indications of digital regrowth are seen (arrows). Scale in millimeters. Middle, Same hindlimb seen in left portion of figure and the amputated contralateral hindlimb. Animal is now thirty-eight days old (thirty-two days of regeneration); the lost and basal portions of three toes are clearly visible. Right, Hindlimbs of animal seen in other two portions of figure, pictured at sixty-nine days of age (sixty-three days of regeneration). Scale in millimeters.

regeneration of atypical legs (Fig. II-15). In other words regeneration of limbs in mammals demands strong destruction and dedifferentiation of tissues in stumps just as observed in tadpoles or frogs.

Skorobogatova (1951, 1951a) has shown in biochemical experiments that the intensity of autolysis and the ability to split tissue proteins in stump limb removal in axolotles remained at high level for forty-eight days after the operation, while in mammals (rats, mice) three to seven days after the operation they return to normal values. The ability of mammals to destruction and dedifferentiation of limb tissues after limb removal is also considerably less than in tailless amphibia. All these results confirm our conclusion (Polezhaev, 1935, 1936, 1948, 1968) that the loss of ability to regenerate limbs during the progressive adaptive evolution is correlated with the decrease in ability for destruction and dedifferentiation of corresponding mesodermal tissues. The artificial stimulation of these processes can lead to partial or complete recovery of the latter. Knowledge of this correlation is very important, since it permits the beginning of a new round of experiments concerning the recovery of the ability to regenerate in different tissues and organs in various mammalian species and ultimately in man.

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III

RECOVERY OF THE ABILITY TO LIMB REGENERATION IN AXOLOTLES AFTER X-RAY IRRADIATION

FORMULATION OF THE PROBLEM

The limbs of urodeles (axolotles, newts and salamanders) are most popular models for studies of regeneration. These organs show very high regeneration ability after simple amputation. Furthermore, such regeneration can be completely suppressed when the limbs are irradiated with a suitable dose of x-rays (Butler, 1933, Brunst, 1937, 1950, 1960, 1965; Trampusch, 1959, 1964; Polezhaev, 1966, 1966a etc). The dose necessary for such suppression depends on the species, as well as on the age of the animal and irradiation conditions. For eight to twelve-months-old axolotles the necessary dose is about 7000-10000 rad.

The ability to regenerate is suppressed for a long period of time, but the general anatomic and histological structure of irradiated tissues and organs as well as their function are maintained as normal. Irradiation probably leads to the alterations of the cells molecular structure (DNA etc.) as well as to a change in their morphogenetic abilities. The first experiments describing the suppression of regeneration ability by irradiation were conducted a long time ago with the larvae of ambystoms, axolotles and newts (Butler, 1933; Lichko, 1934; Brunst and Sheremetyeva, 1935; Puckett, 1936). These investigations lead to the formulation of the basic question—whether the ability to regenerate lost as a result of irradiation can be recovered? The attempts to answer this question can be classified into a number of groups or periods.

REGENERATION AFTER THE TRANSPLANTATION OF NON-IRRADIATED TISSUES TO THE IRRADIATED LIMBS

A new approach to the investigation of this problem began with the work of Umanski (1937), who introduced the method of the transplantation of nonirradiated tissues onto the irradiated extremities of axolotles which were unable to regenerate. Later similar investigation was independently conducted by Thornton (1942) who worked with the newt *Triturus viridiscens*. This author was unaware about the earlier work of Umansky. In these experiments nonirradiated cuffs of the skin, pieces of the muscle or skeletal elements from the limbs or the tail were transplanted into the irradiated limbs. Since homoplastic transplantation in amphibia goes well, grafts were usually taken. After the grafting the limbs were removed so that the transplant remained in the nonamputated part. This resulted in the regeneration which occurred at the expense of transplanted nonirradiated tissues. If nonirradiated limb tissues were transplanted to the irradiated extremity, limb regeneration was observed. If nonirradiated tail tissues were transplanted, this resulted in the regeneration of tail-like organs.

The results of Umansky (1937) were repeatedly confirmed by himself and by a number of other investigators. However the interpretation of these results by different authors was different. Umansky and several other investigators (Thornton, 1942; Luther, 1948; Trampusch, 1951; 1958, 1958a; Stinson, 1963, 1964, 1964a, 1964b; Lazard, 1959, 1959a, 1965, 1965a, 1967) concluded from these experiments that the ability of irradiated tissues to regenerate was lost irreversibly and the only source of regeneration was represented by transplanted nonirradiated tissues. An additional support for this conclusion came from experiments on injection of dissociated nonirradiated cells from axolotl tissues to the irradiated and amputated limbs of other axolotles (Skowron and Rogusski, 1958; Rogusski, 1961; Lagan, 1961; Polezhaev, 1959). The type of regenerated organ was determined by the origin of injected nonirradiated cells.

In contrast to these observations, some other authors who repeated the experiments of Umansky with some modifications concluded that *irradiated tissues* could also be involved in the regeneration, initiated by the transplanted nonirradiated grafts. This might

indicate that the ability to regenerate lost after irradiation could be recovered. For example, Pukhalskaya (1940) transplanted skin cuffs from the irradiated limbs of black axolotles to the nonirradiated limbs of white axolotles. After the amputation of limbs at transplant regeneration occurred and pigmented tissues of the irradiated graft took part in the regeneration process. Liozner (1941, 1947) transplanted nonirradiated pieces of muscle and skeleton from the axolotl tail to the irradiated limbs of other axolotles. After the amputation of the limbs on the grafts, tail-like organs regenerated, which contained, however, the skeletal elements of the limb. Similar results were obtained by Sidorova (1949). In another paper (Sidorova, 1951) she irradiated axolotl limbs with 3.500 rad of x-rays, transplanted unirradiated pieces of heart, lung and liver to these irradiated limbs and then amputated the limbs on the graft. As a result the regeneration of typical or atypical limbs was observed. In these experiments the transplants could not serve as the source of cellular material for the regeneration but they could *stimulate* the regeneration of the irradiated limb tissue. It can be argued however that the dose of 3.500 rad could not completely inhibit the regeneration ability of axolotl limb, since it is known (Brunst, 1937; Umansky, 1937) that the necessary dose for axolotles is about 7.000-10.000 rad. Blakher (1952) transplanted nonirradiated tissues of triploid axolotles to the irradiated limbs of usual diploid axolotles. After the amputation and subsequent second limb amputation on the transplant the resulting blasthems were studied cytologically. It was found that these blastema contained both diploid and triploid cells. In other words, irradiated cells of the recipient participated in the regeneration. Trampusch (1959) transplanted unirradiated skin cuffs from the black axolotles to the irradiated limbs of white axolotles. After the amputation on the transplant in most cases (eighty-one cases) the regeneration of the grey limbs was observed. These limbs contained pigmented tissues of the transplant. In three cases the limbs were white. The author suggests that this demonstrated the ability of irradiated recipient tissues to regeneration. This, however, is disputed by Rose (1964). He suggests that the result can be explained by the migration of unirradiated epidermal cells from the proximal regions of the white limb. These cells are located above the trans-

planted nonirradiated black skin and they gave rise to the blastema Rose, Quastler and Rose (1955) irradiated limbs in newts and removed the irradiated skin. The limbs responded by covering with epidermis from the limb base, followed by regeneration. The authors concluded that epidermis formed the blastemic cells. We would suggest however that it was more probable that unirradiated epidermis contributed to the recovery of ability of irradiated limb tissues to regenerate. Similar explanation appears to be valid for the results of Lazard (1959, 1965, 1965a), who studied regeneration of axolotl irradiated limbs after the homotransplantation of nonirradiated abdominal skin, testicular tissue or embryonic tissues in these limbs. She suggested that in these experiments graft tissues served the source of regeneration but the direction of their development was changed by the hypothetical "field" existing in the recipient limb. We suggest that it is more probable that in these experiments the transplants lead to the recovery of the regeneration ability of irradiated limb tissues.

Thus the method of transplantation of nonirradiated tissues to the irradiated organ opened the way to a number of interesting studies. These studies lead to different conclusions. The irradiated organs can regenerate a) only at the expense of grafted nonirradiated tissue or b) both irradiated and nonirradiated tissues can serve as a source of regeneration. This latter possibility will be considered in more detail on the basis of newer experiments.

EXPERIMENTS ON THE RECOVERY OF REGENERATION ABILITY IN AXOLOTL EXTREMITIES AFTER X-RAY IRRADIATION

In attempts to recover the regeneration ability of axolotl limbs after x-ray irradiation we tried to apply some methods which we were using earlier for the recovery of organ regeneration ability lost in ontogeny—or phylogeny (Polezhaev, 1959, 1966, 1966a, 1968; Polezhaev and co-workers, 1960-1964; Teplits, 1962, 1964, 1964a; Tuchkova, 1964-1969). A number of morphological, biochemical, histological, histochemical and cytological methods were used (Polezhaev, 1966, 1966a, 1968).

The dose of irradiation varied between 6.000 and 10.000 rad without filter. Both hind extremities were irradiated to their base, the body was shielded by the lead plate 4 mm in thickness, and

amputation of feet of both irradiated extremities was conducted. The dose 7.000 rad led to the necrosis of fingers, 8.000 rad resulted in necrotic lesions of fingers and foot, 10.000 resulted in necrotic alteration of the foot in 75-100 per cent of cases. The age of animals varied between eight and twelve months and time between irradiation and amputation was between seven and twenty-one days. After irradiation with the dose 7.000-10.000 rad and subsequent amputation regeneration was absent in 93 per cent of cases, the wound healing proceeded smoothly (Fig. III-1). It

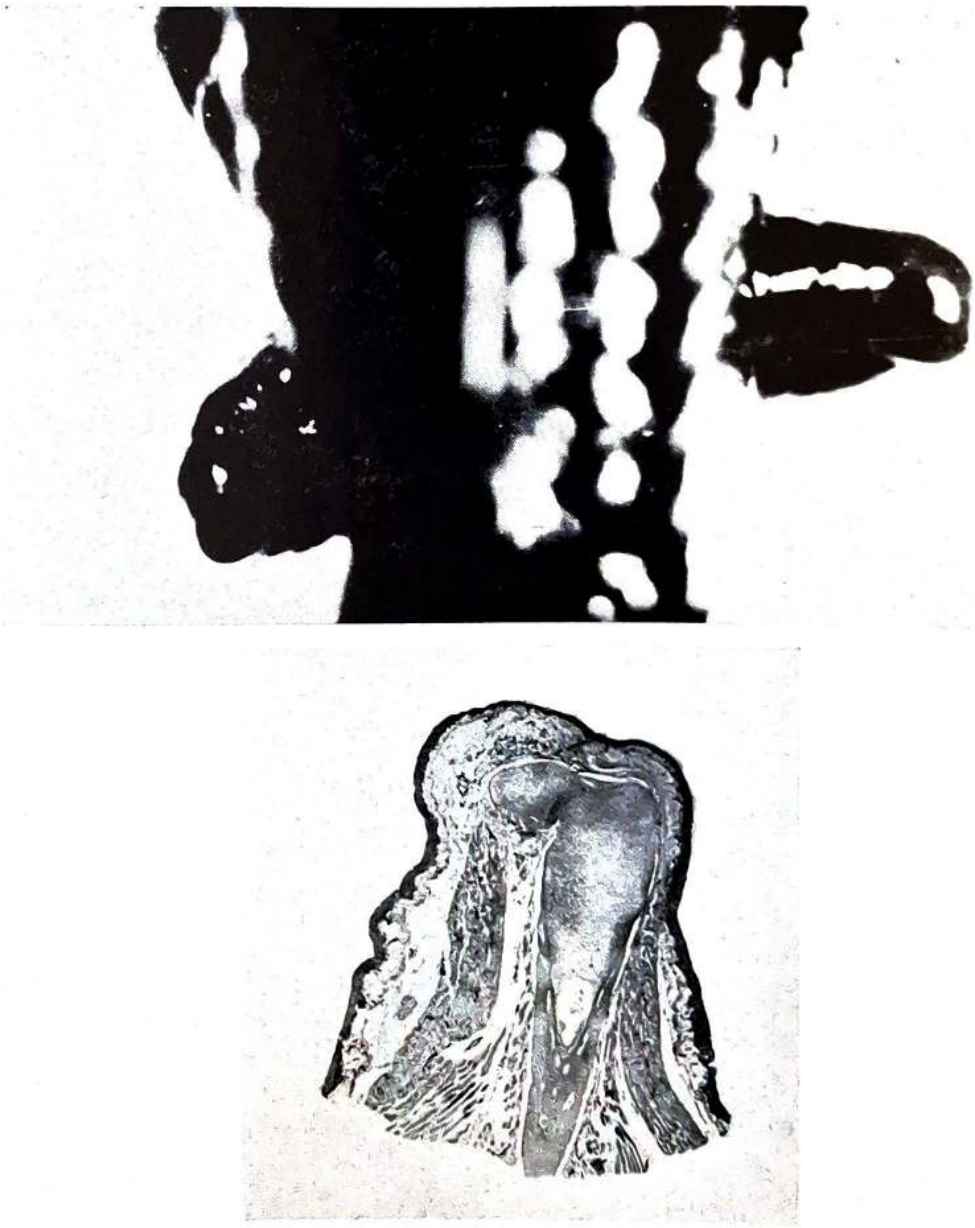

Figure III-1. No regeneration of limbs in axolotl after irradiation with 7.000 rad and amputation (three months after operation). Top, General view of the limbs. Bottom, Longitudinal section of the limb.

Brunst, 1937, 1950, 1960, 1965; Trampush, 1959, 1964; Polez- should be mentioned however that sometimes the formation of lasting x-ray-induced ulcers was observed on the healed wound one to two months after the amputation.

After the irradiation and amputation homogenates of leg muscles obtained from the nonirradiated black axolotl were injected to the right hind leg of the white axolotl. As a result gray limbs

Figure III-2. Regeneration of one limb in axolotl after irradiation (7.000 rad) and amputation of both limbs, homogenate of the rat thigh muscle being injected into one of the stumps. Top, General view of the limbs. Bottom, Longitudinal section of the regenerated limb.

regenerated in about 30 per cent of the cases on the wound surface of either right injected or left noninjected leg. These regenerates originated from the dissociated cells of unirradiated homogenates (Polezhaev, 1959). These results are similar to those obtained by Skowron and Rogusski (1958). On the other hand, if irradiated right hind extremities were injected with homogenates prepared from rat thick muscle, rat liver or rat testis, we also observed the regeneration of either right injected or left noninjected hind legs in 30 to 35 per cent of the cases (Fig. III-2).

The remaining part of the irradiated extremity was long and consisted of both femur and shank and this made rather improbable the suggestion (Rose, 1964; Schmidt, 1968; Thornton, 1968) that cells of nonirradiated tissues including cells of the pelvis area which migrated to the wound after the puncture with the syringe needle could be the source of such regeneration. The per cent of regeneration on injected and noninjected extremities was the same. Regenerated limbs had normal structure and contained skeleton, muscles, connective tissues, skin, nerves, blood vessels and pigmented cells. These regenerated limbs could not regenerate from injected rat cells, since they had the structure of axolotl extremities not the rat ones and it could be concluded that the regeneration was due to the recovery of regeneration ability of limb elements suppressed by x-ray irradiation.

Freezing of rat muscles used for homogenate preparation to $-18°$ abolished their capacity to induce the recovery regeneration ability suppressed by x-ray irradiation, boiling and sterilization lead to marked decrease of the activity. Fresh homogenates of the skin were inactive.

Furthermore the activity of various subcellular components was studied. To that purpose clean nuclei, DNA, ribosomal RNA and protein were isolated from rat liver cells. The activity of the so-called milk acid protein (Perov, 1947) was also tested.

It was found that nuclei and DNA were inactive, while RNA and protein were active. When RNA and protein were injected together into the irradiated animals, the activity to stimulate regeneration was lost. Only freshly prepared high polymer RNA was active, after storage in a refrigerator for one and a half to two months the activity was lost, commercial RNA also was inactive.

According to the literature the irradiated tissues irreversibly lose their ability to regenerate and secondary amputation does not change it. Our experimental results, however, necessitate the revision of this viewpoint. If the frequency of limb regeneration after the x-ray dose of 7.000-10.000 rad was about 7 per cent after the second amputation of distal piece of the stump 4 to 5 mm long three months after the first operation, regeneration frequency was about 17 per cent. In experiments reported by Stinson (1964) the regeneration after the first amputation was observed in 4 per cent of the cases, after the second operation regeneration frequency was 12 per cent.

If homogenates, protein or RNA are injected into one of the irradiated extremities only one of them regenerates after the amputation in about 33 per cent of the cases. If, without repeating injections, all regenerated and nonregenerated limbs are amputated a second time, as distal as possible, the frequency of regeneration attains 70 or even 100 per cent (Fig. III-3). Usually this results

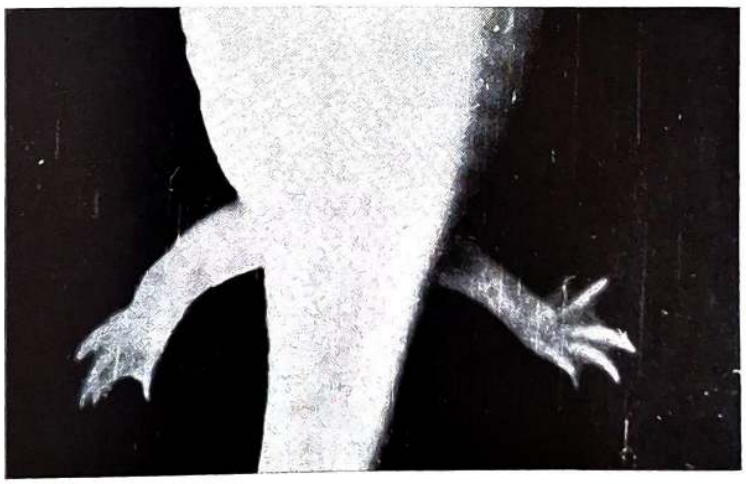

Figure III-3. Regeneration of both limbs in axolotl after irradiation (7.000 rad), homogenate of the rat thigh muscle being injected into one of the stumps, amputation and second amputation three months later.

in regeneration of both paired limbs, even in those cases when they did not show any regeneration after the first amputation (e.g. after the injection homogenates prepared from rat muscles were frozen at $-18°$). It can be concluded that two to three months after the injection there is a considerable rise in regeneration ability. We can speak therefore of the *increasing effect*. The importance of this increasing effect can be demonstrated from the following experi-

ment. Both hind extremities of axolotl were irradiated with the dose 10.000 rad and after twenty days the right leg received six daily injections of RNA. The animals were divided into two groups. The first group was operated on immediately after the end of injections, in the second group the amputation was performed two months later. The frequency of regeneration in the first group was 33 per cent, while in the second group it was 100 per cent.

In another experiment the extremities of the first case were amputated twenty days after irradiation. In the second case amputation took place six months after irradiation. The frequency of regeneration was 13 per cent and 23 per cent respectively. It can be concluded that there is certain spontaneous recovery of regeneration ability after x-ray irradiation, but trauma contributes to this recovery to a greater extent than the factor of time.

In the experiments of Tuchkova (1966) the extremities of young axolotles were irradiated with 6.000 rad and then amputated;

Figure III-4. The restoration of regenerative capacity of axolotl limbs by additional trauma. Both hindlimbs were totally irradiated with 7.000 rad, twenty days later, both feet being amputated. The left one was the control and the right, experimental. After amputation, the stump was repeatedly damaged with pincers for 5 days. Limbs regenerated in 47 per cent of the cases. The result of the experiment three months after amputation (Tuchkova, 1966).

mesodermal tissues of the stump were repeatedly traumatized with forceps branches. These irradiated stumps regenerated with the frequency of 50 per cent while control paired limbs on the other side of the animal did not regenerate at all (Fig. III-4). In another experiment both feet of the animal were removed twenty days after the irradiation with 8.000 rad; the left extremity served as a control and minced irradiated limb muscles were transplanted to the skin cuff of the right extremity. No regeneration was observed in the control, while in the experimental limb it was observed in many

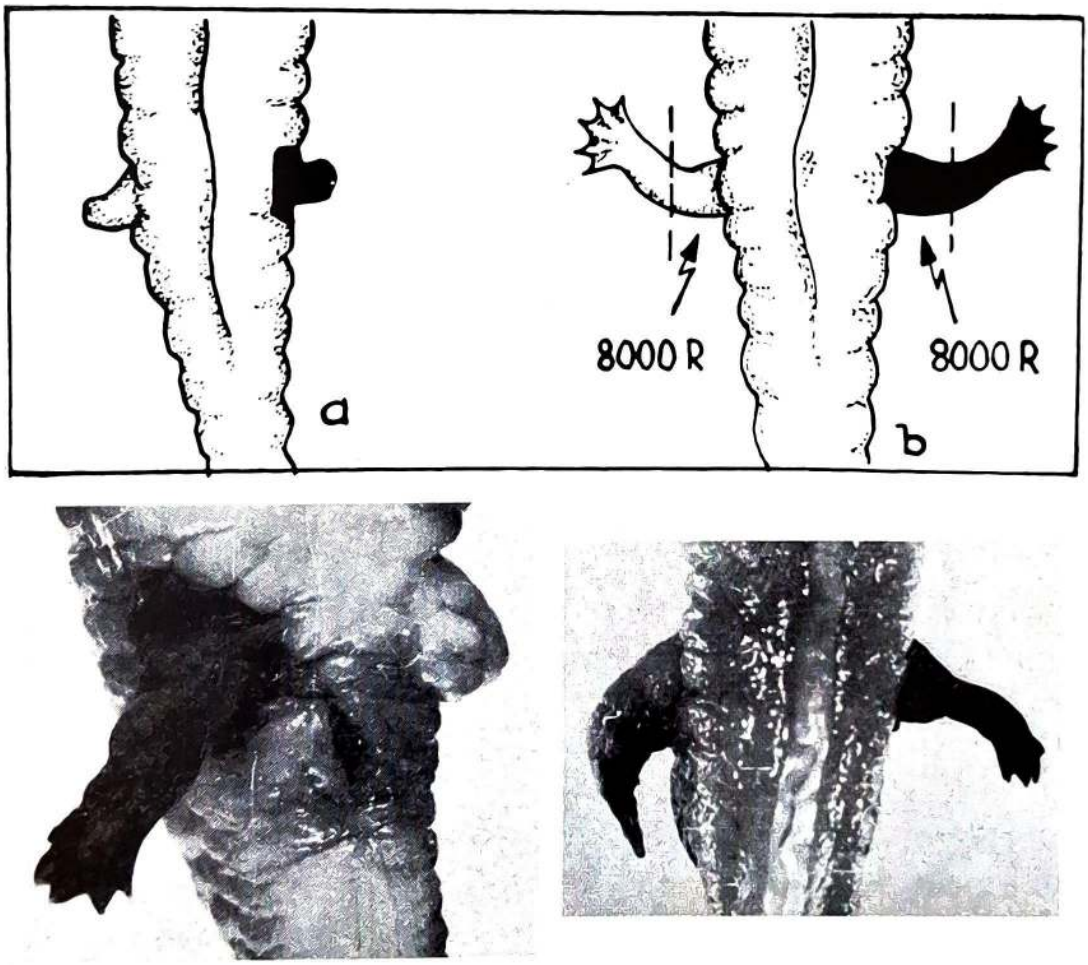

Figure III-5. Regeneration of black axolotl limb, transplanted to white axolotl after irradiation (7.000 rad) and RNA injections. Top, Course of operation. a, A part of the thigh of the hindlimb was grafted into the site of completely amputated hindlimb of the white axolotl. Simultaneously, the left hindlimb was amputated at the level of the transplantate b.: b, Regeneration of the transplanted and own axolotl limbs after total irradiation of the limbs with 8.000 rad (indicated by a broken arrow) and their amputation twenty days later. Bottom left and right, The result of the experiment. A black limb develops from the black stump of the white axolotl.

cases; it was probably due to the action of destroyed irradiated tissues. It can be inferred that trauma has a powerful effect on the irradiated tissues facilitating the recovery of their ability to regenerate.

It has already been mentioned that some authors (Rose, 1964; Thornton, 1968; Schmidt, 1968) are inclined to suggest that the recovery of regeneration ability is due to the migration of nonirradiated cells through the irradiated tissues of the organ residue. To resolve this possibility the following experiment was conducted (Tuchkova, 1967). Extremities of black axolotles were transplanted to the white animals after removal of their own right hind legs. After the transplant grafting unoperated and transplanted extremities were irradiated with 8.000 rad, and twenty days later feet of both extremities were removed. Right extremities received five daily injections of RNA. This resulted in the recovery of regeneration ability and *black* extremities (not white or gray) regenerated in white animals (Fig. III-5). Thus transplantation occurred at the expense of *local irradiated tissues* not of migrated recipient cells. These results are confirmed by experiments where thymidine (^{3}H) labeled cartilage or limb homogenate were transplanted to the irradiated newt extremities (Desselle, 1968).

MORPHOLOGICAL AND BIOCHEMICAL INVESTIGATIONS

Most investigators hold the view that the inhibition of organ regeneration ability after irradiation is due to the impairment of cell proliferation mechanisms (see Brunst, 1950, 1960). However results of morphological experiments conducted by Tuchkova (1964-1969) lead us to another conclusion. It was demonstrated that irradiation did not affect the destruction ability of irradiated tissue, but suppressed their ability to *dedifferentiation* which preceded the burst of mitotic activity. After the removal of nonirradiated extremities the following pattern of events takes place. Mesodermal tissues become dedifferentiated, the cells release from the tissues and become morphologically uniform. They look like mesenchyme embryonic cells with large clear nuclei, containing big nucleoli. The cytoplasm is moderate in appearance and rich in RNA (Fig. III-6). After the initial accumulation period, intensive mitotic divisions are observed. Irradiation abolishes the dedifferentiation

Figure III-6. Regeneration of blastema cells of nonirradiated limb in the axolotl. (Magnification:ocular 7, objective 90).

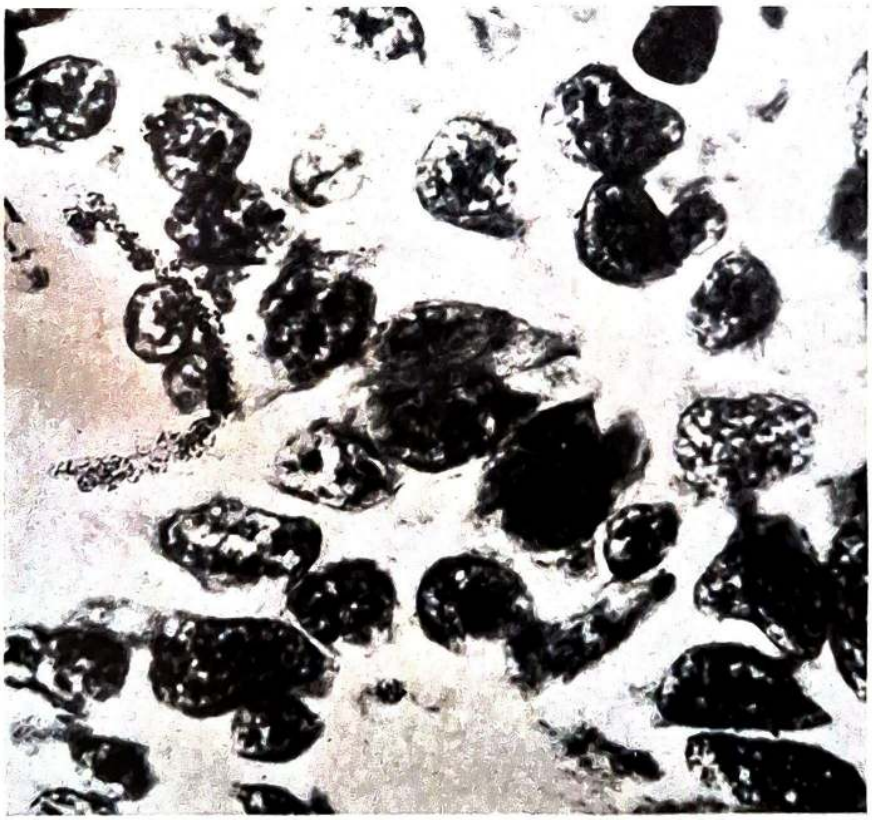

Figure III-7. "Rough" and connective tissue cells arising in the area of amputation surface of limbs in axolotl after irradiation with 8.000 rad. Regenerative capacity is suppressed. (Magnification: ocular 7, objective 90).

phase. Instead of embryonic-like blastema cells "rough" cells are formed. Their nuclei contain irregular chromatin bodies, thick nuclear envelope, nucleoli are not visible and cytoplasm is poor in RNA (Fig. III-7). Mitotic activity of these cells is four times lower

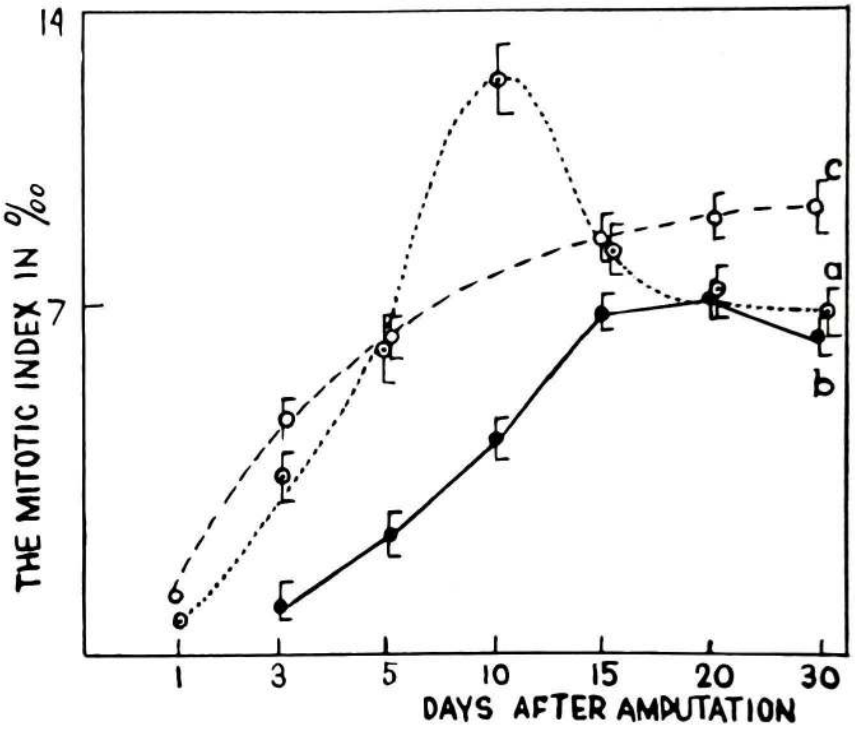

Figure III-8. Mitotic activity of regenerating epithelium after amputation of limbs in the control, after irradiation with 8.000 rad, after irradiation (8.000 rad) with restoration of suppressed regenerative capacity by RNA injection (Tuchkova, 1967); a—Regeneration of unirradiated limbs; b—Healing of irradiated limbs after amputation; c—Restoration of regenerative capacity suppressed by x-irradiation.

than that of blastemic cells (Figs. III-9). The results of radioautographic experiments using thymidine (H^3) and uridine (H^3) (Tuchkova, 1969, 1969a) demonstrated that the ability to DNA and RNA synthesis in the mesodermal tissues irradiated extremities is significantly decreased (Fig. III-11, 13, 15, 17). It is interesting in this connection that the ability to DNA and RNA synthesis in regenerating epidermal tissue of extremities is not effected by irradiation to any considerable extent (Fig. III-8, 10, 12, 14, 16). The recovery of ability to regeneration is accompanied by the recovery of ability to dedifferentiation, morphogenesis, mitotic activity and ability to high DNA and RNA synthesis in mesodermal tissues. Rose (1965) also came to the conclusion that irradiation

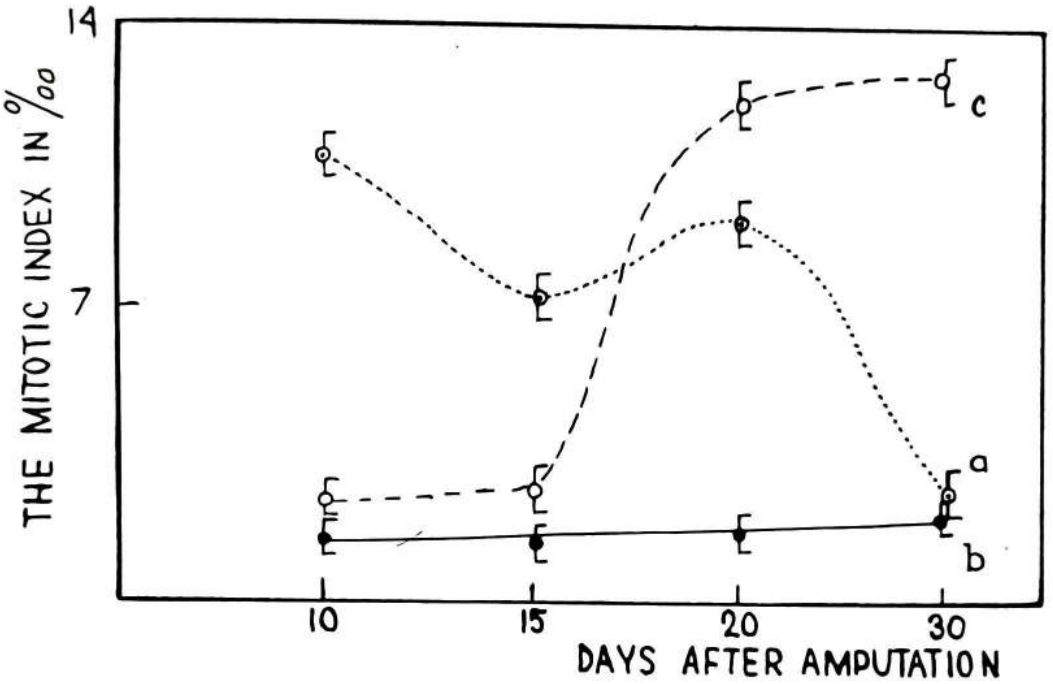

Figure III-9. Mitotic activity of blastema cells, "rough" and connective tissue cells after amputation of axolotl limbs without irradiation, after irradiation with 8.000 rad, after irradiation (8.000 rad) with restoration of the regenerative capacity by injection of RNA (Tuchkova, 1969); a—Regeneration of unirradiated limbs; b—Healing of irradiated limbs after amputation; c—Restoration of regenerative capacity suppressed by x-irradiation.

inhibits the dedifferentiation phase of regenerating extremities tissues in amphibia.

These results indicate that irradiation leads to considerable but still incomplete inhibition of nucleic acid synthesis and mitotic activity in mesodermal tissues. It seems probable that irradiation leads to such alteration of cell molecular structure (conformation?) which inhibits their ability to dedifferentiation and only later to inhibition of nuclec acid synthesis and mitotic activity. The recovery of regeneration ability suppressed by x-ray irradiation is due to the recovery of cell molecular structure, which is followed by normalization of its other properties. Other explanations, of course, are also possible.

Results of biochemical experiments performed by Teplits (1962, 1964) have shown that x-ray irradiation of intact extremities in axolotles leads to a reproducible decrease of nucleic acid content in their tissues. The minimal values were observed approximately one month after irradiation. Later nucleic acid content increased somewhat, but never attained the values of nonirradiated controls

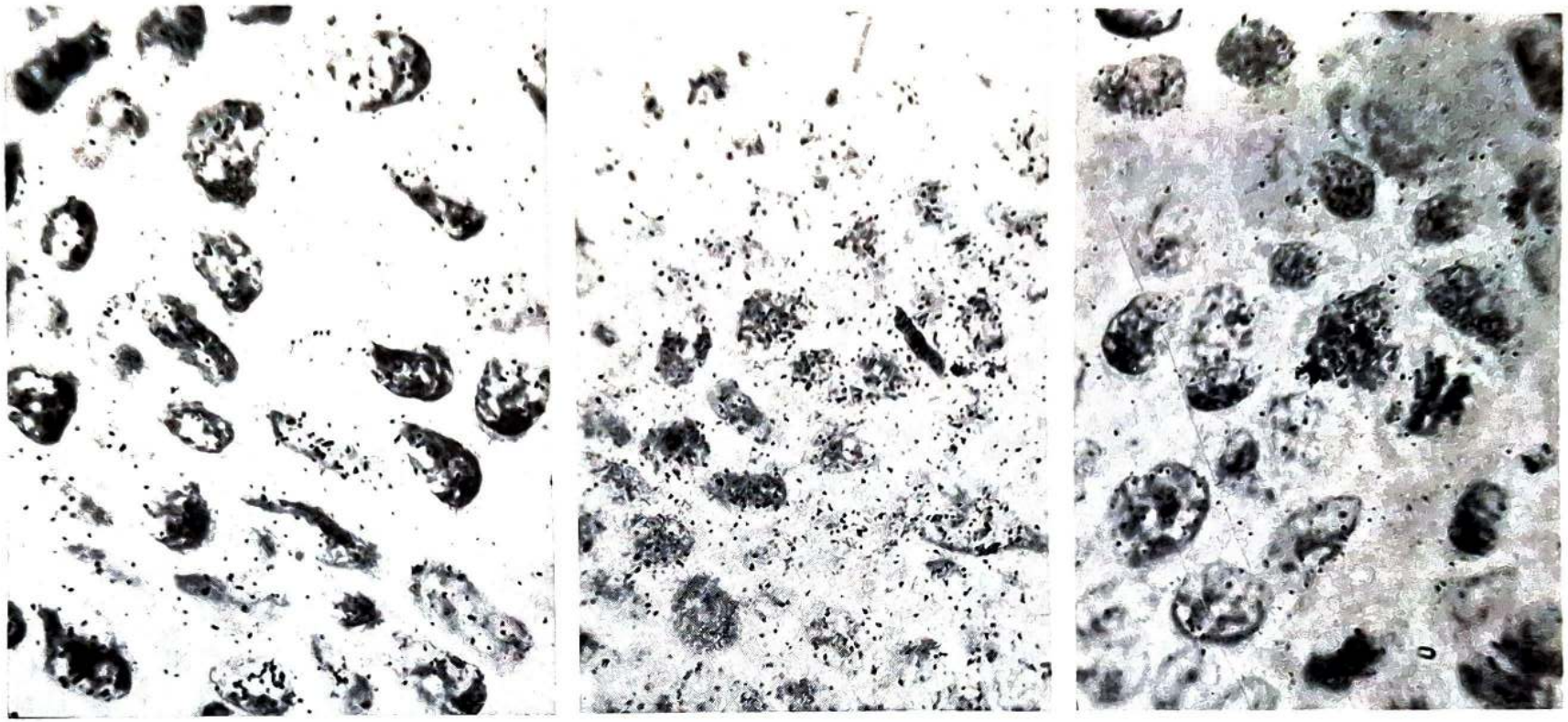

Figure III-10. Incorporation of H^3-uridine in regenerating epithelial cells seventeen days after amputation. H^3-uridine was injected intraperitoneally twenty-four hours before fixation of tissues (H^3-uridine - 2 μC/g). Oc.10, ob.60 x (Tuchkova, 1969). Top, Epithelium of unirradiated limbs; Middle, Epithelium of irradiated limbs; Bottom, Epithelium of irradiated limbs in the experiment wth restoration of suppressed regenerative capacity by RNA injection.

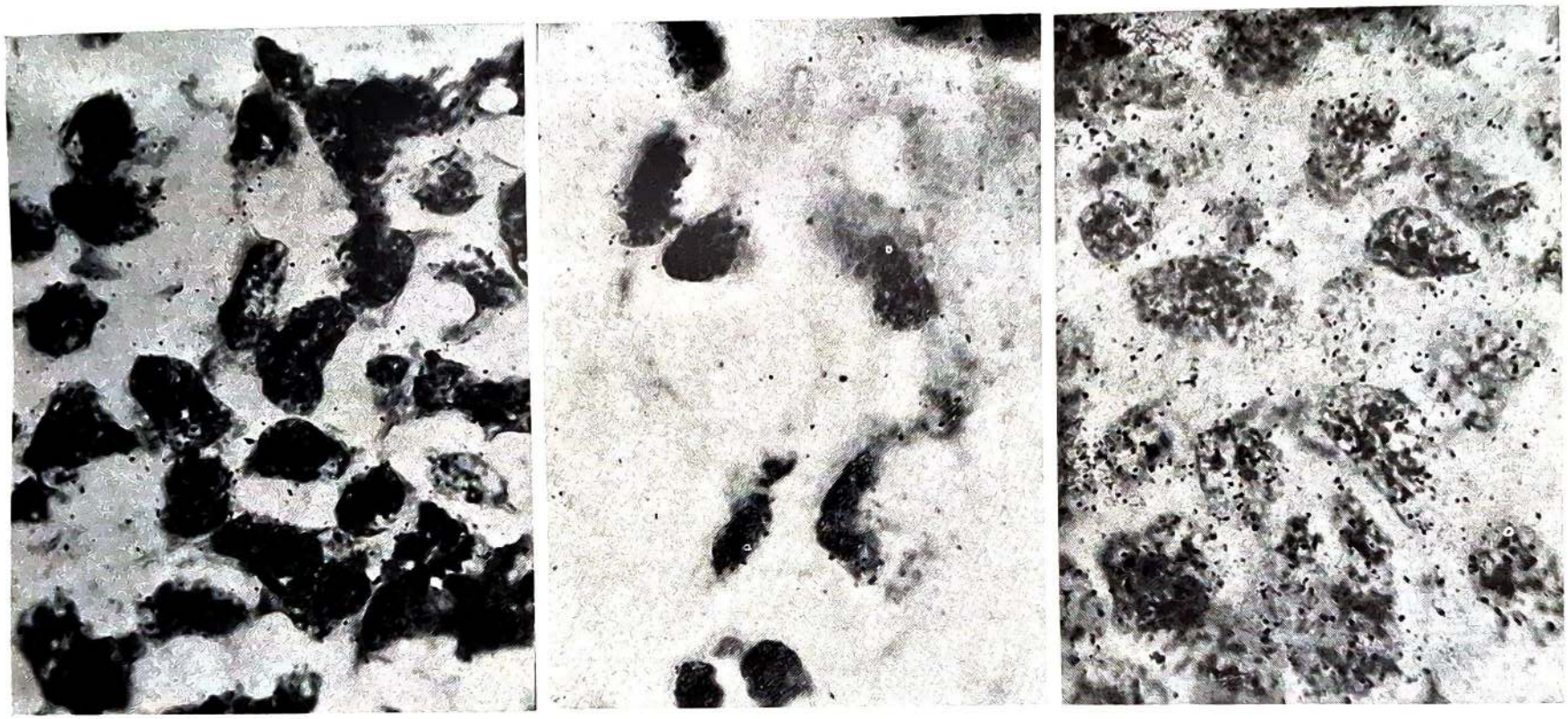

Figure III-11. Incorporation of H^3-uridine in regenerating blastema cells, "roug'ı" and connective tissue cells in axolotl seven days after amputation of limbs. Ocular 10, objective 60 x (Tuchkova, 1969). Top, Blastema cells of unirradiated limbs; Middle, "Rough" and connective tissue cells of irradiated limbs; Bottom, Blastema cells of irradiated limbs in experiment with restoration of suppressed regenerative capacity.

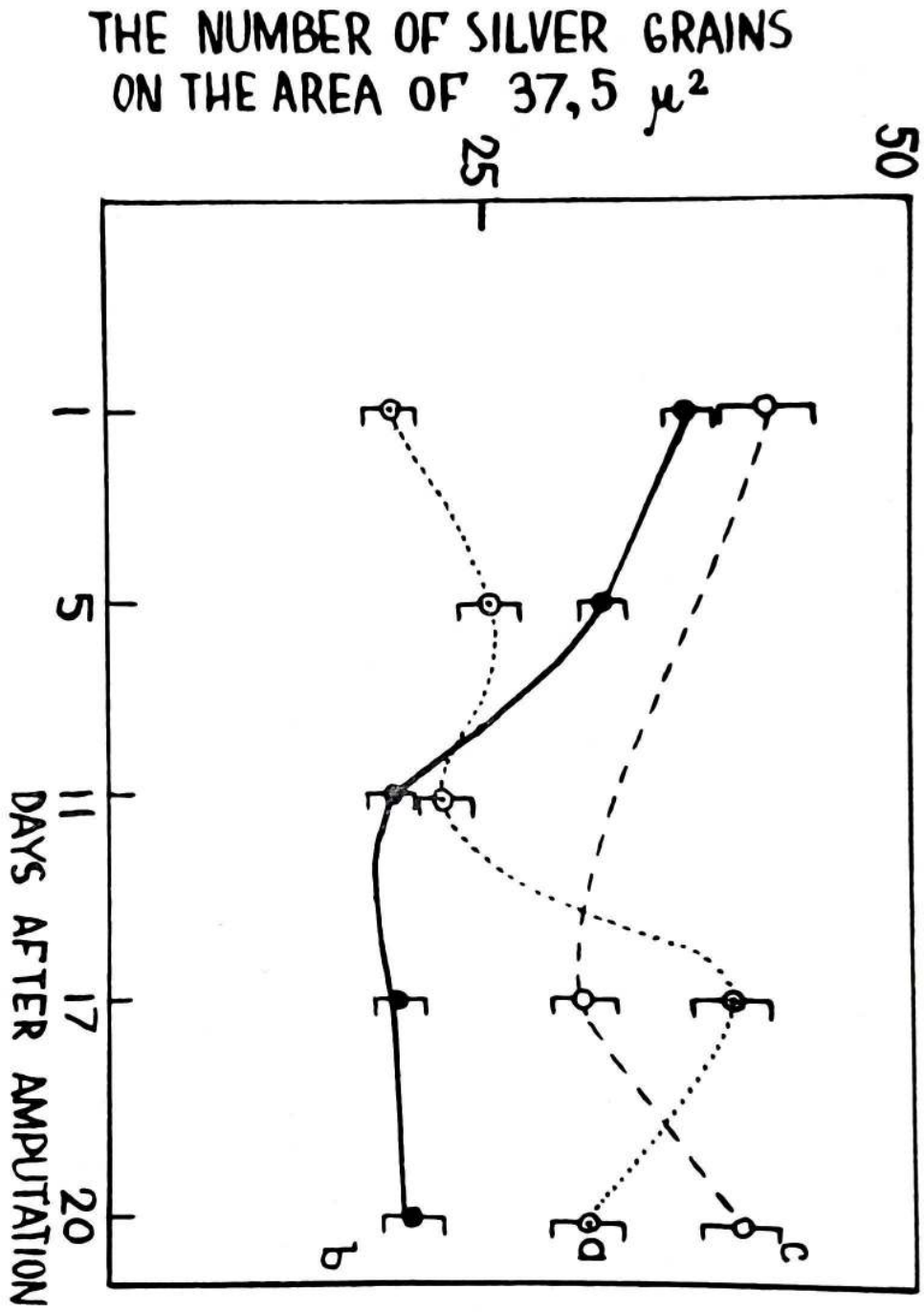

Figure III-12. Changes in the intensity of H^3-uridine incorporation into regenerating epithelial cells after limb amputation of axolotl without irradiation, after irradiation with 6.000 rad, after irradiation (6.000 rad) with restoration of the suppressed regenerative capacity by RNA injection (Tuchkova, 1969); a–Regeneration of unirradiated limbs; b–The wound healing after amputation of the irradiated limbs; c–Restoration of the regenerative capacity, suppressed by x-irradiation.

(Fig. III-18, 19). The injection of exogenous RNA to the irradiated animals did not change DNA and RNA content in these tissues. If the extremities were irradiated and then removed, the nucleic concentration remained low. But if exogenous RNA was injected to such operated animals, DNA and RNA concentration in tissues increased and attained the normal level. At the same time the regeneration ability of extremities was recovered (Fig. III-20, 21). It is remarkable that in this case the x-ray-induced ulcers were not observed. In controls they were present in a relatively large per cent of cases on healing wound surfaces of operated extremities.

Kirrmann (1968) has shown that irradiation decreases nucleic acid content and mitotic activity in the chick embryo gut cells grown *in vitro,* and exogenous RNA addition results in the return of these parameters to normal values. His results confirm our own observations.

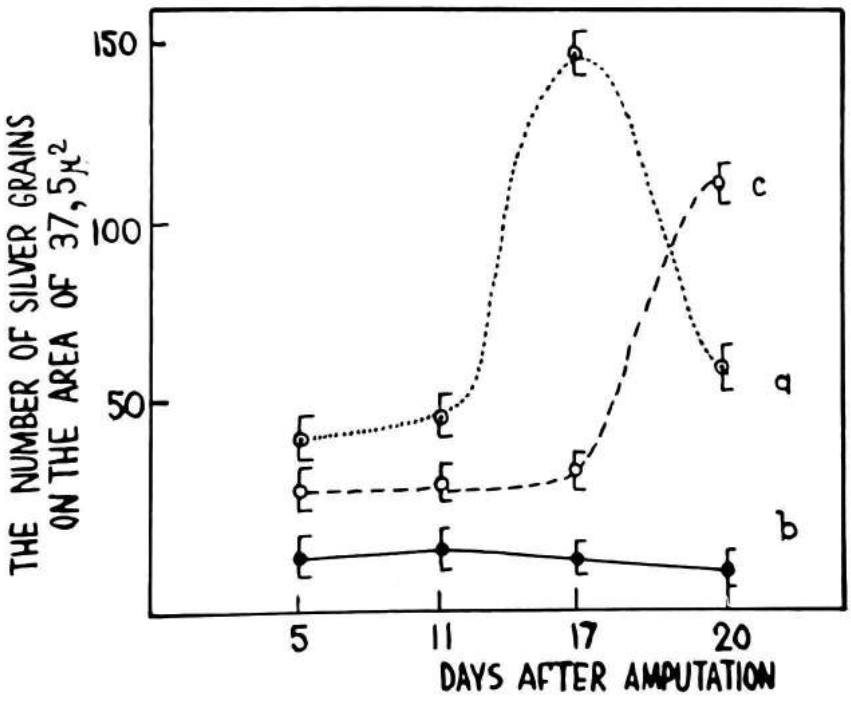

Figure III-13. Changes in the intensity of H^3-uridine incorporation into blastema cells, "rough" and connective tissue cells after limb amputation without irradiation, after irradiation with 6000 rad, after irradiation (6000 rad) with restoration of the suppressed regenerative capacity by RNA injection (Tuchkova, 1969); a–Regeneration of unirradiated limbs; b–Wound healing after amputation of irradiated limbs; c–Restoration of regenerative capacity suppressed by x-irradiation.

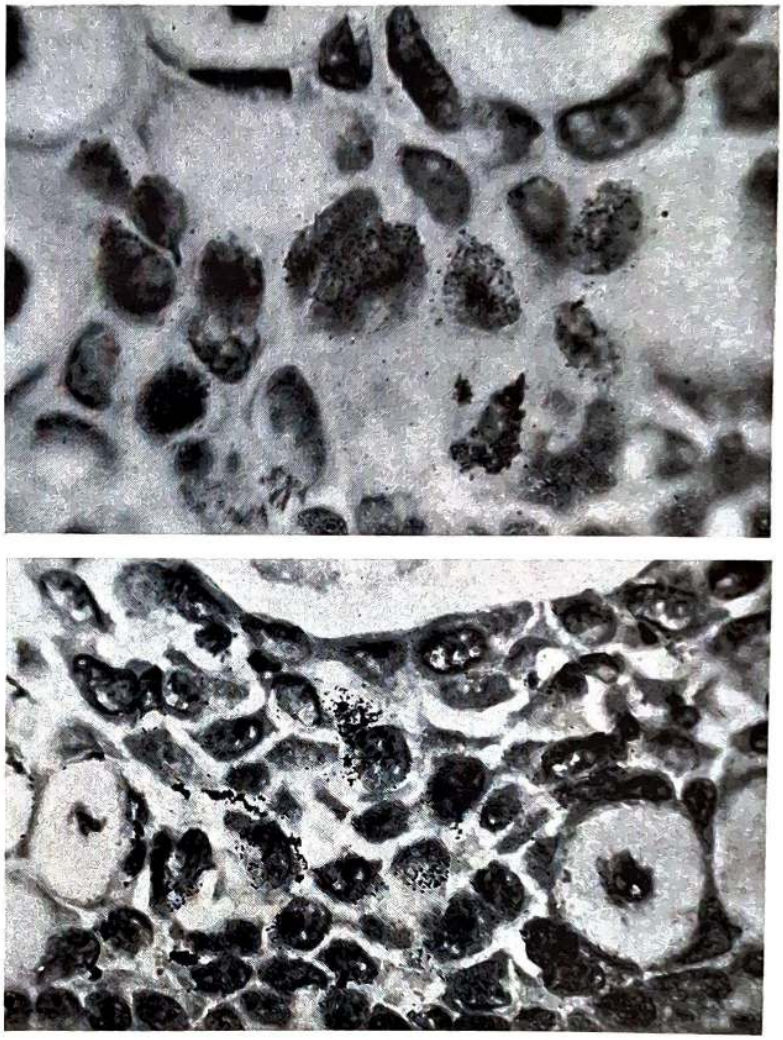

Figure III-14. H^3-thymidine incorporation into the regenerating epithelial cells five days after amputation (magnification: ocular 10, objective 60) (Tuchkova, 1969). Top, Epithelium of nonirradiated limbs; Bottom, Epithelium of irradiated limbs.

In the course of limb regeneration during the destruction and dedifferentiation phase DNA and RNA content in tissues of organ residue is decreased to reproducibly low levels. In contrast, in regenerating blastema tissues nucleic acid content is increased. (Teplits, 1964a) (Fig. III-22, 23). Probably protein biosynthesis in regenerating tissues is related in some way with protein synthesis in tissues before operation. The loss of limb regeneration ability in ontogeny or phylogeny of animals is due to the lowered ability to destruction and dedifferentiation of basic mesodermal tissues. The loss of regeneration ability in amphibia after x-ray irradiation is due to the impairment of dedifferentiation ability of these tissues. The destruction phase in these circumstances is not affected. If

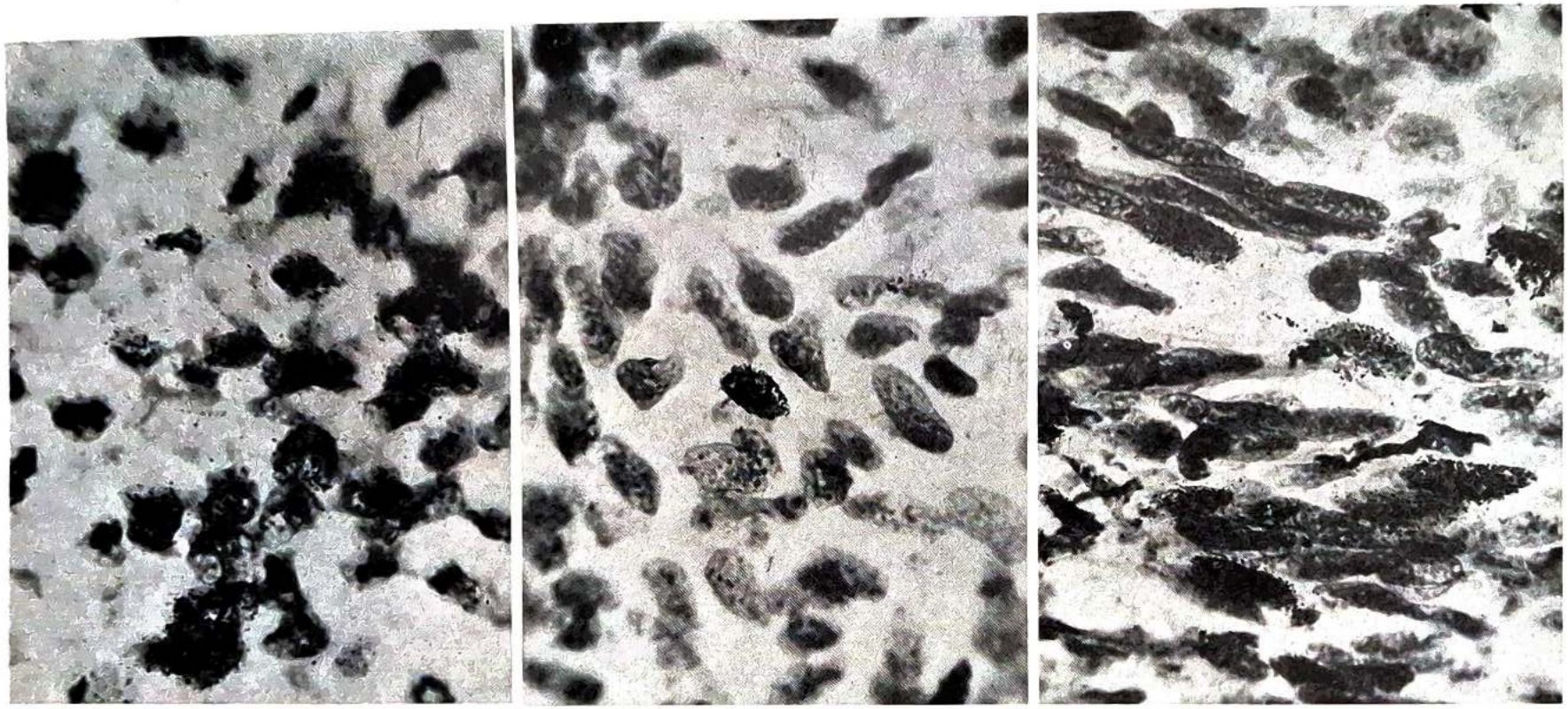

Figure III-15. H^3-thymidine incorporation into blastema cells, "rough" and connective tissue cells (magnification: ocular 60, objective 90). H^3-thymidine was injected six hours before fixation of the tissues (Tuchkova, 1969). Top, Blastema cells of nonirradiated limbs, twenty days after amputation; Middle, "Rough" and connective tissue cells, underlying the epithelium in the wound area, twenty days after amputation; Bottom, Blastema cells (twenty-five days after amputation) of irradiated limbs under the restoration of suppressed regenerative capacity by RNA injection.

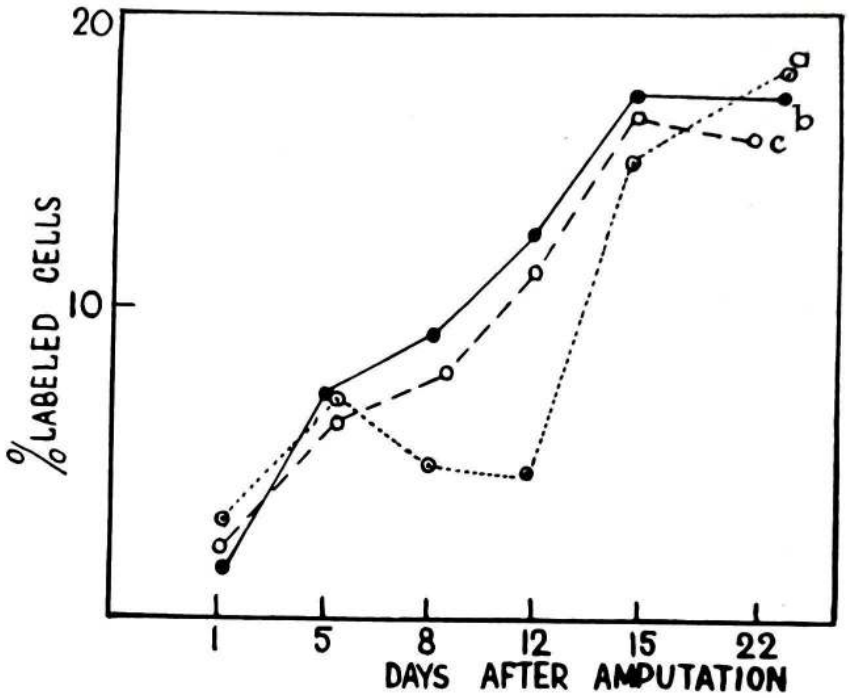

Figure III-16. Changes in the percentage of H^3-thymidine labelled cells in regenerating epithelium after amputation of limbs without irradiation, after irradiation with 6.000 rad, after irradiation (6.000 rad) with restoration of suppressed regenerative capacity by RNA injection (Tuchkova, 1969a); a–Regeneration of unirradiated limbs; b–Wound healing after amputation of irradiated limbs; c–Restoration of regenerative capacity, suppressed by x-irradiation.

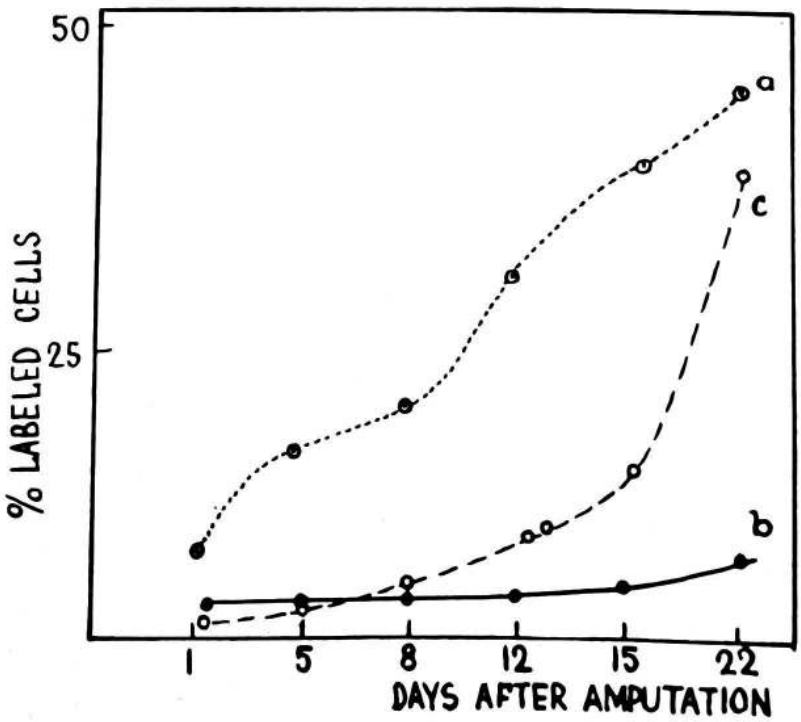

Figure III-17. Changes in the percentage of H^3-thymidine labelled blastema cells, "rough" and connective tissue cells after limb amputation without irradiation, after irradiation with 6.000 rad, after irradiation (6.000 rad) with restoration of suppressed regenerative capacity by RNA injection (Tuchkova, 1969a); a–Regeneration of unirradiated limbs; b–Wound healing after amputation of irradiated limbs; c–The restoration of the regenerative capacity, suppressed by x-irradiation.

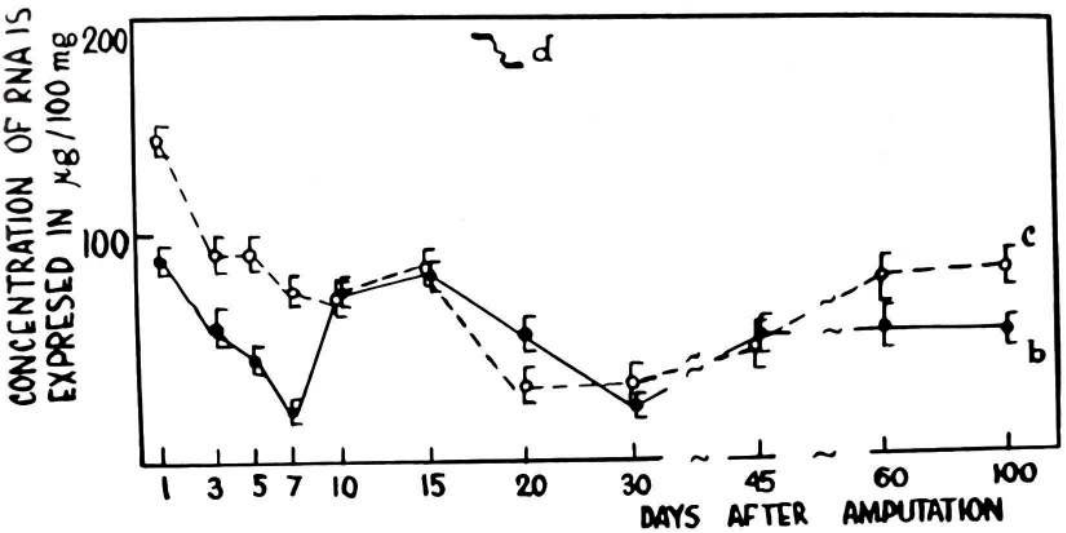

Figure III-18. RNA concentration in unamputated limb tissues of the axolotl without irradiation, after x-irradiation, after x-irradiation and RNA injection (Teplits, 1964a); b–Tissues of limb after irradiation; c–Tissues of limb after irradiation and RNA injection; d–Tissues of intact limb.

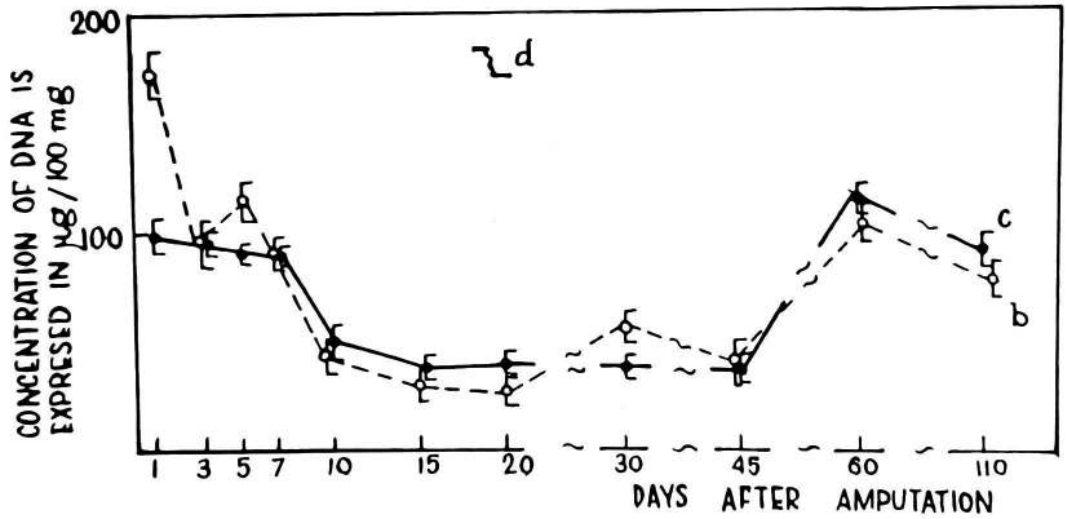

Figure III-19. DNA concentration in unamputated limb tissues without irradiation, after x-irradiation, after x-irradiation and RNA injection (Teplits, 1964); b–Tissues of limb after irradiation; c–Tissues of limb after irradiation and RNA injection; d–Tissues of intact limb.

the destruction is augmented in some way, the ability to dedifferentiation and regeneration are recovered. We can thus conclude that in axolotles limb regeneration ability suppressed by x-ray irradiation can be recovered. X-ray treatment suppresses the ability of mesodermal tissues to dedifferentiation, decreases their ability to nucleic acid synthesis, as well as their mitotic activity. When regeneration ability is recovered all other cellular characteristics

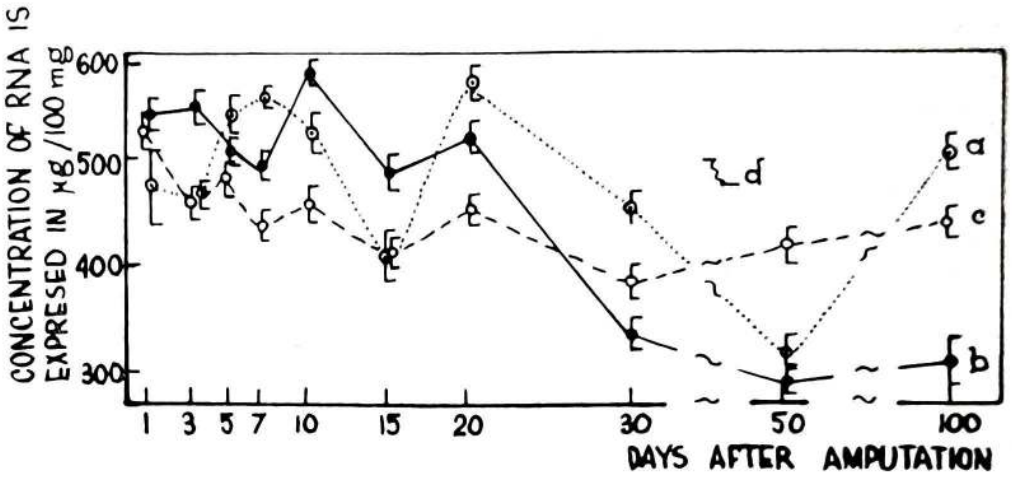

Figure III-20. RNA content in limb tissues of the axolotl without irradiation, after x-irradiation, suppressing regenerative capacity, and its experimental restoration (Teplits, 1962); a–Tissues nonirradiated limb in the course of regeneration (control); b–Limb tissues after irradiation, amputation and suppression of regeneration; c–Limb tissues after irradiation, amputation, RNA injection and regeneration; d—Tissues of an intact limb.

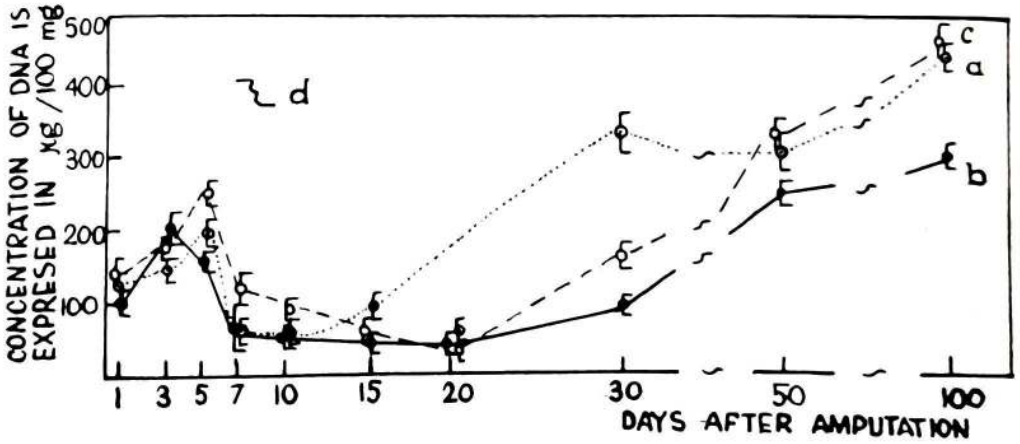

Figure III-21. DNA content in limb tissues of axolotl without irradiation, after x-irradiation, suppressing regenerative capacity and its experimental restoration (Teplits, 1962); a–Tissues of nonirradiated limb in the course of regeneration (control); b–Limb tissues after irradiation, amputation and suppression of the regenerative capacity; c–Limb tissues after irradiation, amputation, RNA injection and regeneration; d–Tissues of an intact limb.

return to normal values. This, however, does not exclude other explanations.

For example, it can be suggested that irradiation does not impair certain cells, where DNA structure and ability for dedifferentiation survive this treatment. But I do not think that this hypothesis is

more attractive than the above one. All the cells of a given organ, for example, axolotl limb receive the same dose of x-rays. After the amputation neither dedifferentiation, nor regeneration take place. Injection of exogenous RNA, protein fractions or homogenates, result in the recovery. Experiments conducted by Kirrmann (1968) *in vitro* lead to the same result. In the latter case no unimpaired cells were present. Nevertheless we cannot completely exclude this interpretation of some other possibility.

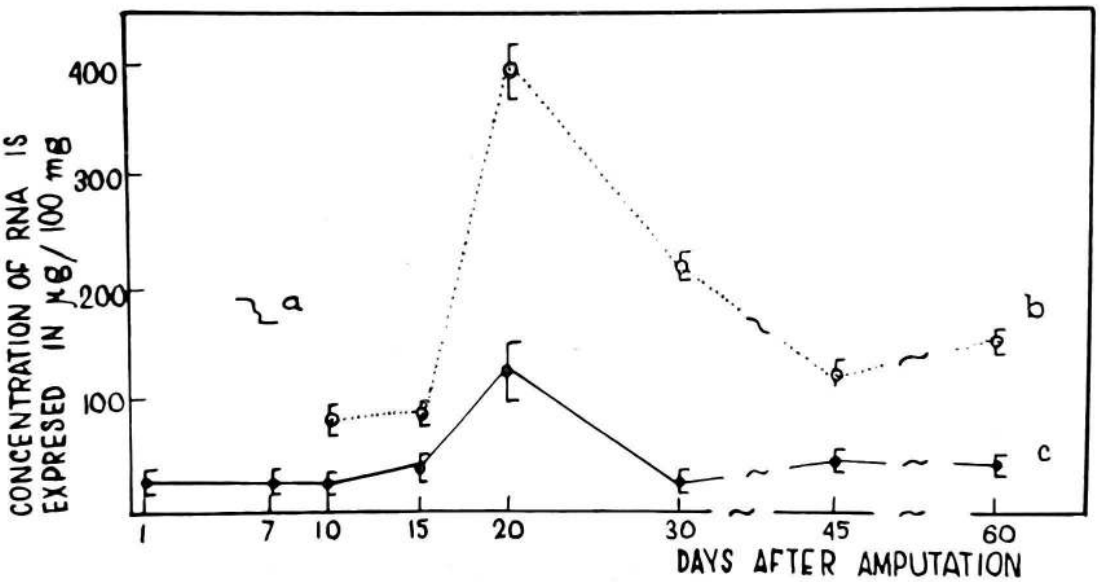

Figure III-22. Determination of RNA content in blastema and in old stump tissues of axolotl limbs (Teplits, 1964a); a–Tissue of blastema; b–Old tissues of the stump; c–Tissues of an intact limb.

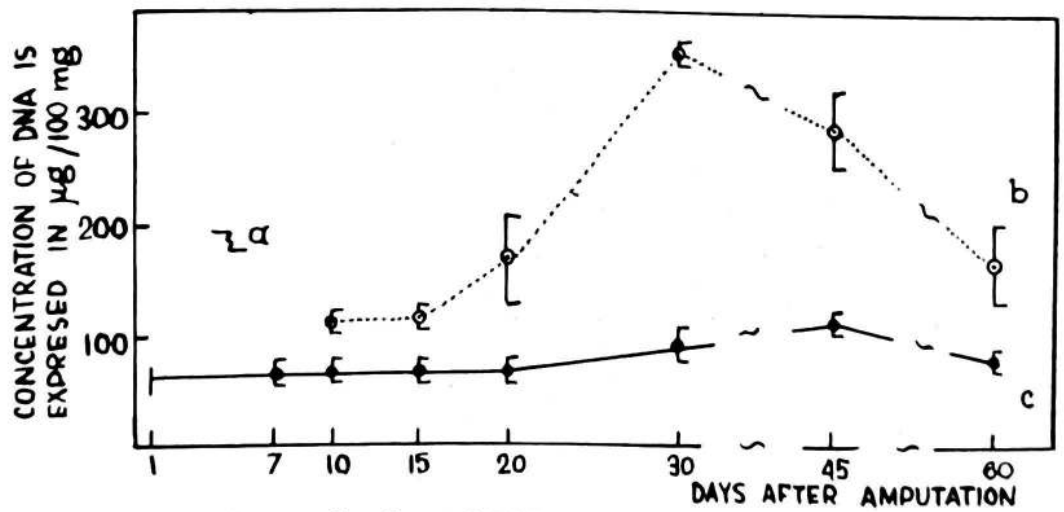

Figure III-23. Determination of DNA concentration in blastema and in old tissues of the limb stump in axolotl (Teplits, 1964a); a–Tissues of blastema; b–Old tissues of the stump; c–Tissues of an intact limb.

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IV

RECOVERY OF REGENERATION ABILITY OF SKULL VAULT BONES IN MAMMALS AND MAN

FORMULATION OF THE PROBLEM

If our concept concerning the determinative factors governing recovery of organs and tissues in animals is correct, it is tempting to use it for the attempts to regenerate parts of those organs which are not regenerated under normal conditions in mammals. N.N. Burdenko Institute of Neural Surgery suggested to us some years ago the development of biological methods of skull defect substitution in humans. This presented an interesting possibility and we began working in this direction. Skull vault bones in humans, as well as in dogs and some other mammalian species, usually do not regenerate after partial removal, and damaged regions are healed with fibrous scar tissue. Under such conditions the brain remains unprotected from possible mechanical damage. It should be noted in this connection that in cases of certain traumas, as well as malignant lesions of brain envelopes and the skull, large pieces of skull bones are frequently removed and they must be substituted in some way. The literature dealing with this problem is quite extensive (Marchand, 1901; Grekov, 1901; Ozerov, 1909; Nemilov, 1940; Leibson, 1959; Sirola, 1961; Matveeva, 1962; Potanina, 1963 etc).

Tubular bones of mammals including man usually regenerate well, the source of regeneration is the periosteum. The external surface of flat skull bones (ossa investintia) is covered with periosteum, their inner side is layered with dura mater, which is regarded by a number of anatomists and histologists as a second periosteum (Korning, 1931, etc.) Nevertheless, when pieces of skull bone are removed, and the pericranium and dura mater are left, bone regeneration does not occur. A number of surgeons substituted the skull defects by bone pieces but frequently the trans-

plants eroded and defects opened again. The last twenty years are characterized by the increasing uses of alloplastics for the substitution of skull defects—this means the substitution by nonliving materials such as polymethylmetacrylate, tantal, horn, etc. But even such foreign materials are not quite inert and harmless for the contacting tissues. Therefore a search for suitable biological methods for the substitution of skull bone defects remains very important. We tried to approach this problem from the standpoint of the theory of regeneration and suggested four new biological methods, which permit skull vault bone regeneration in mammals and in man.

DEVELOPMENT OF BIOLOGICAL METHODS FOR THE REGENERATION OF SKULL VAULT BONES

The experiments were conducted with mice, rats, rabbits, cats, dogs and other mammalian species. The results obtained are presented in a number of papers (Polezhaev, 1951, 1957, 1959; Matveeva, 1962; Rogal, 1952, 1955, 1956, 1957; Korchak, 1959; Kantorova, 1968, 1968a, etc.). Operations were conducted with narcotized animals under strictly aseptical conditions. Rectangular pieces were removed from the parietal bone, the sizes of these rectangles were 6 x 4 mm for mice, 10 x 5 mm for rats, 15 x 10 mm for rabbits and 45x25 mm for dogs. Dura mater was left and frequently was covered by the isolated pericranium. The experiment lasted as long as two years, more than one thousand animals were operated on.

It has been established that in adult dogs, rats, mice, sheep, pigs and goats, the skull vault bones were not regenerated; damaged regions were covered with a scar. It is known, however, that in adult rabbits and guinea pigs they are capable of regeneration (Polezhaev, 1957; Rogal, 1956; Potanina, 1963).

Furthermore it was found that in young rats a few days old, and in puppies and kittens both one month old, the skull vault bones were capable of completing regeneration (Fig. IV-1). It can be inferred, therefore, that the ability of these bones to regenerate was lost during the ontogenic development of these species. In infants, younger than three years, the skull vault bones also show a certain capacity to regeneration. Biochemical experiments have shown that relatively soft porous skull bones of adult rabbits and puppies which

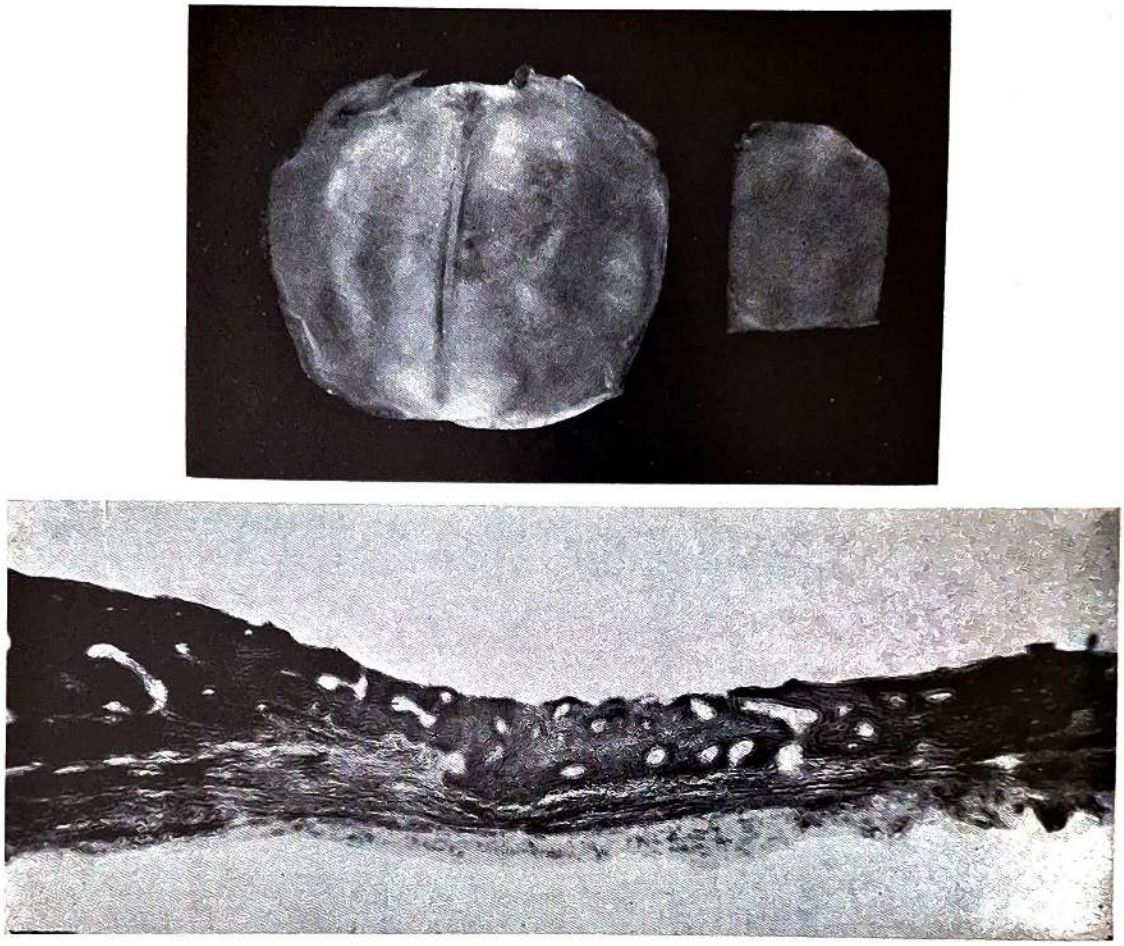

Figure IV-1. Regeneration of calvarium bone in one-month-old cat. Top, Calvarium after regeneration; Removed piece of bone; Bottom, Longitudinal histological section of regenerated bone, one month after operation.

are capable of regeneration can be destroyed relatively easily. This is accompanied by washing away of calcium salts loosely bound to proteins. Dense and hard skull bones of dogs, which are incapable of regeneration are destroyed with much difficulty—the washing of calcium salts firmly bound to tissue proteins is hindered in this case (Korchak, 1959).

Experiments with rabbits have demonstrated that skull vault bones still can regenerate when periosteum is removed from the large area around the bone defect. But they never regenerate when a piece of dura mater is cut out from the damaged region.

When the skull defects in dogs are substituted by skull bone pieces from dog embryos the transplants erode but stimulate the surrounding tissue to complete regeneration (Polezhaev, 1951, 1957). Surgeon Okulova tried this method under clinical condi-

tions in 1955 and obtained complete substitution of skull defect by the regenerated bone.

Another method for the substitution of skull defects, suggested by the author is a method of bone fragment displacement. Bier (1923) suggested an approach for the elongation of tubular bones in humans. He cut them transversally and after a few days pullled them gradually from each other. He suggested that bone destruction products might stimulate the transformation of surrounding connective tissue into the bone. In this way he was able to increase long bone length by 15 cm. We conducted similar experiments with mice and dogs. Again rectangular pieces of the parietal bone were cut out, dura mater was left intact. Then an additional bone strip was cut at one of the edges. After a few days it was displaced into the wound using two silk thread loops leading to the surface of the dog's head. This resulted in complete bone regeneration in the damaged region both in mice and in dogs (Fig. IV-2).

The third method of skull bone regeneration induction made use of the directed alteration of metabolism. The problem here was to stimulate ossification of young, immature connective tissue in the damaged region. This could be done by the increase of calcification. For this purpose certain amounts of calcium lactate, vitamin D and vitamin A were added to the ration of experimental animals. It is known, that vitamin D facilitates the accumulation of calcium salts in the tissues. These experiments were conducted by Rogal (1952, 1955, 1956, 1957) with rats, dogs, sheep and goats. Complete regeneration of the skull bones in all species was obtained. The method is very simple and the treatment is quite physiological. It demands however prolonged administration of calcium salts and vitamin D, and this can lead to sclerosis in aged patients. This is why we continued our search for biological methods of skull defects substitution. The most simple and efficient one was the method of destruction which will be described in the next section.

SKULL BONE REGENERATION INDUCED BY DESTRUCTION

In development of this method we started from the following points:

1. When pieces of bone are removed from the skull vault of mice, rats or dogs, the opened bone marrow cavities are

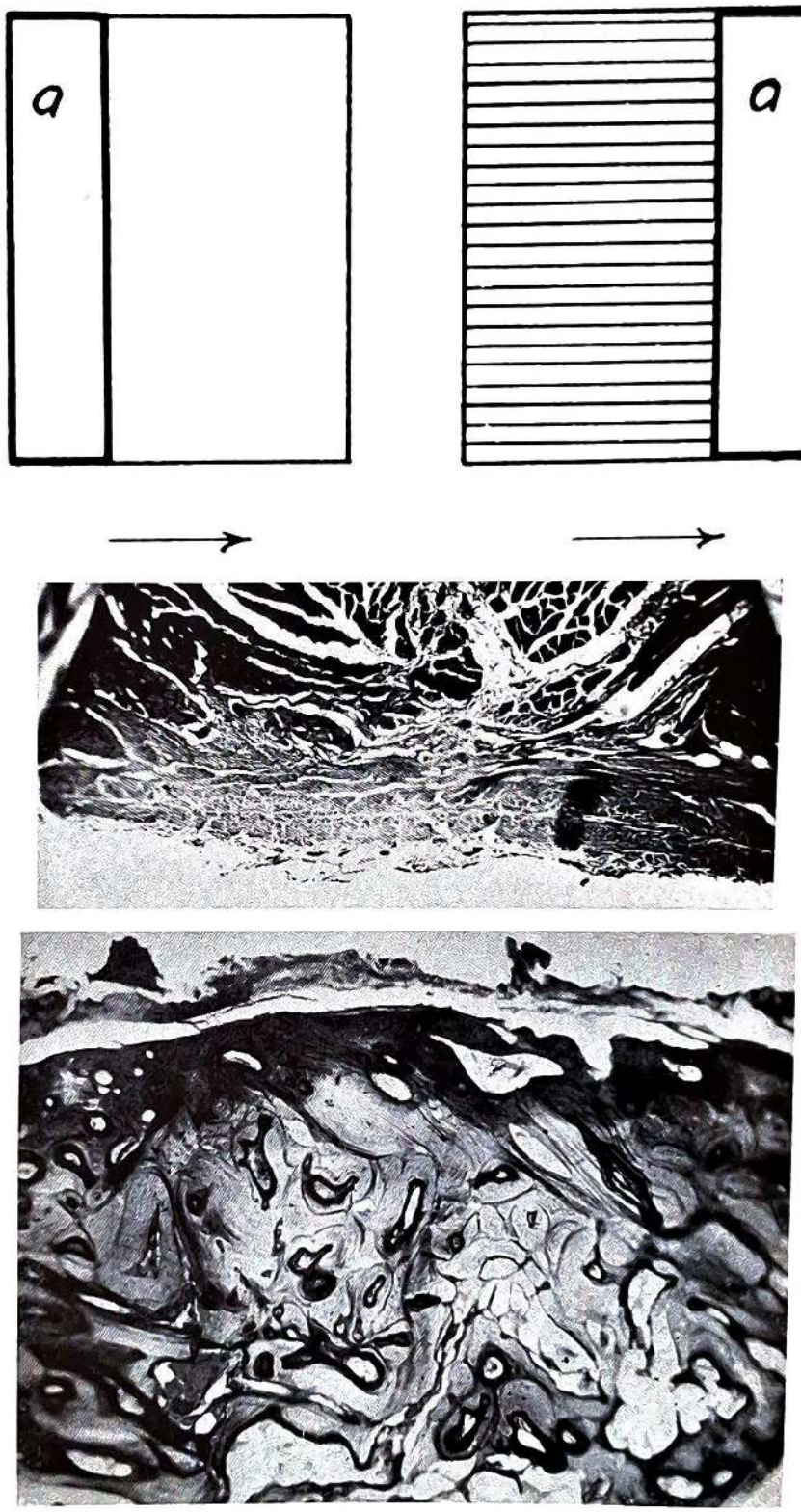

Figure IV-2. Regeneration of calvarium bone induced by dislocation of a bone fragment. Top, Diagram of dislocation of the bone fragment (a) in the damaged area; (right) before dislocation, (left) after dislocation. Middle, Formation of the scar(s) in the damaged area of the scull in adult dog; Bottom, Bone regenerated in the damaged area of the scull after translocation of the bone fragment; 120 days after operation.

plugged by new bone tissue, and certain symptoms of regeneration can be observed in the pericranium. These phenomena *might* be due to the action of bone destruction products, and can be augmented.

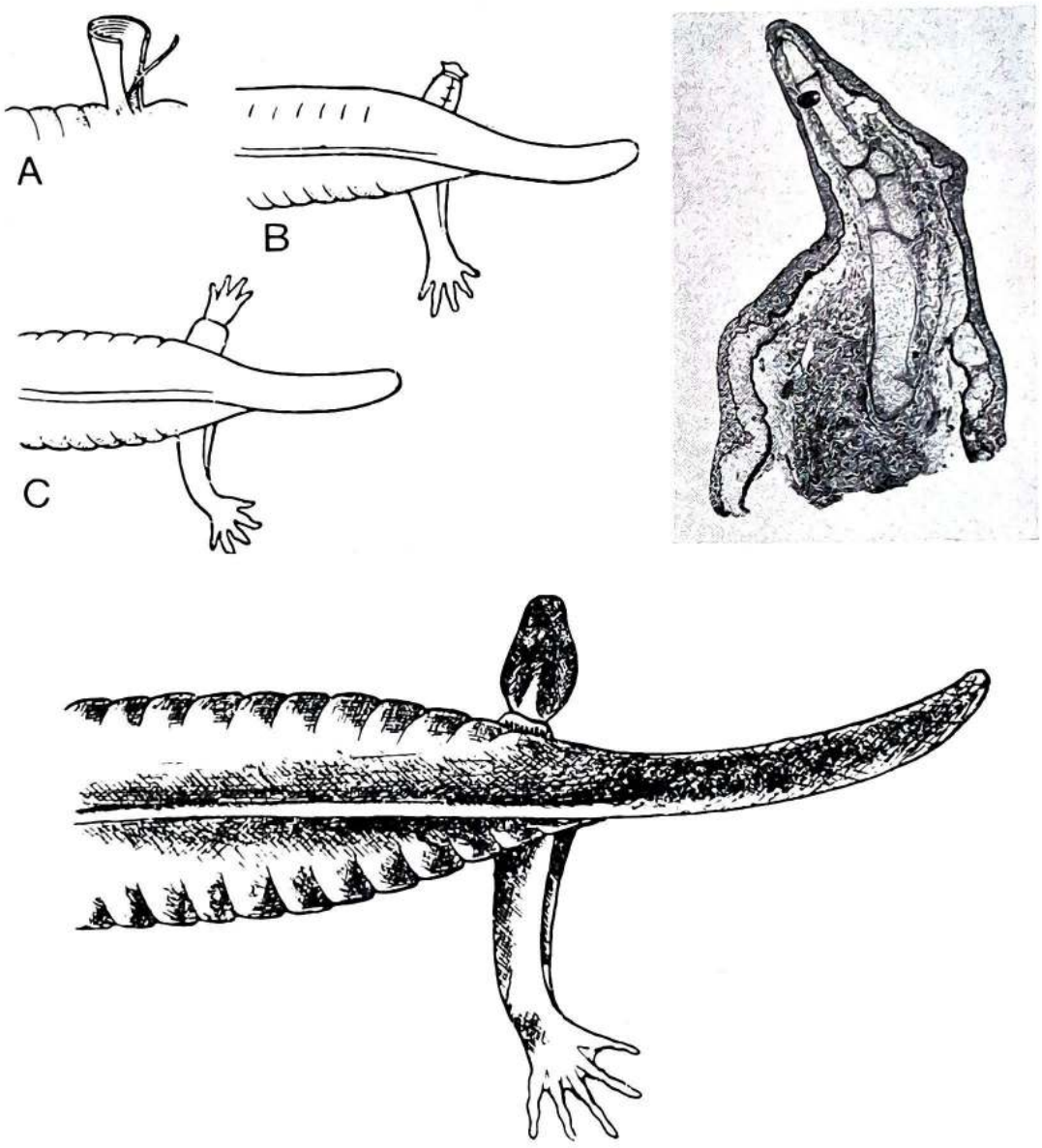

Figure IV-3. Regeneration of organs from destructed tissues in axolotl. Course of operation and its results. Top left, A, Skin flap and nerve were left at the site of amputated limb; B, Minced muscles or muscles, skeleton, connective tissue were grafted into the skin flap; the nerve was led through the implantant; C, Partial amputation of the skin flap with implanted destructive tissues results in regeneration of a typical limb; Top right, Longitudinal histological section through regenerated limb; Bottom, Regeneration of a tail-like organ originated from destructed tissues of mesodermal primordium of the tail grafted into the skin flap of the limb.

2. Strong destruction and dedifferentiation of mesodermal tissues in the limbs of anurans results in the recovery of their ability to regenerate.

3. Destruction of basic mesodermal tissues in axolotl limbs results in complete regeneration of these organs (Polezhaev, 1934, 1936, 1937, 1937a).

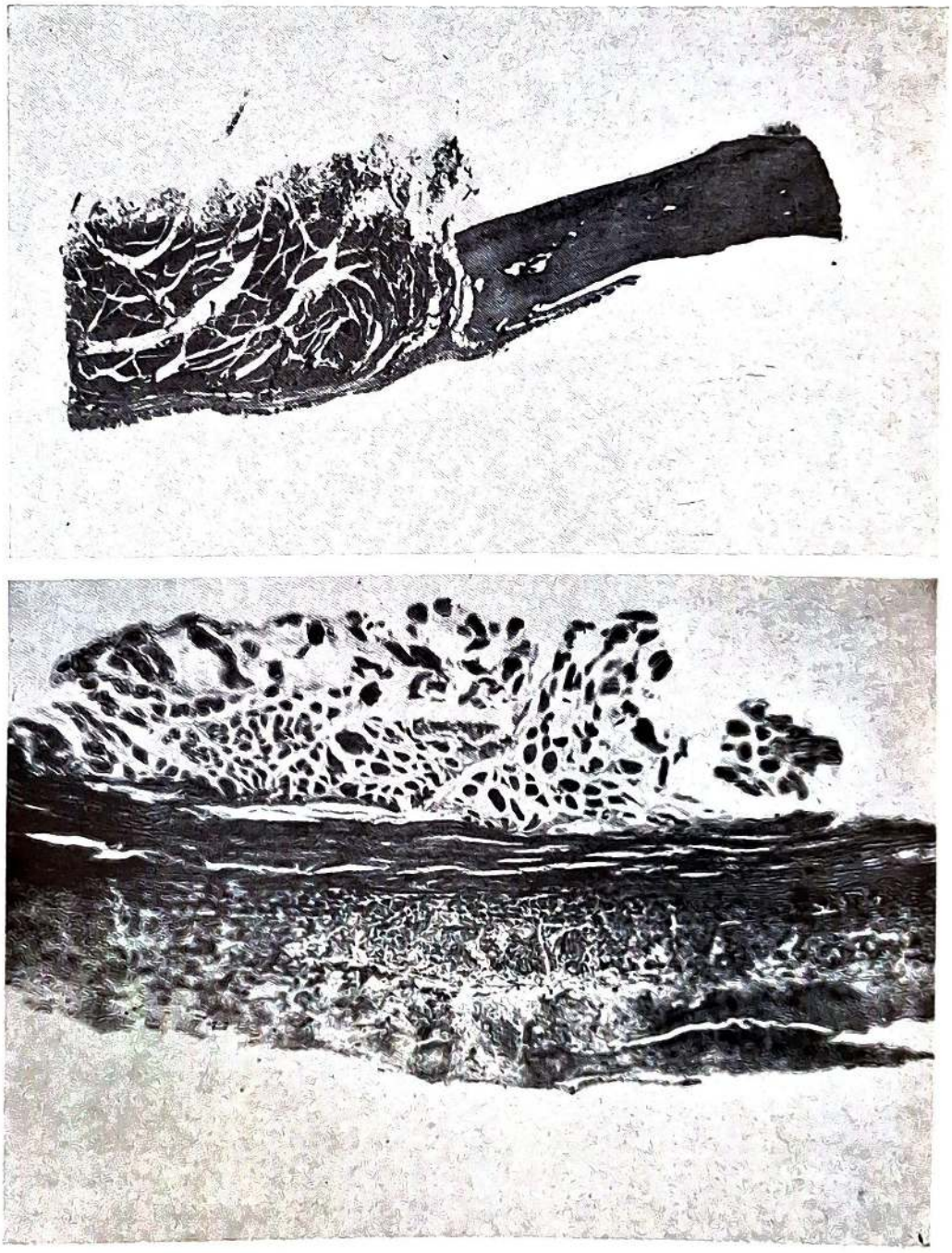

Figure IV-4. No regeneration of bone and filling of destruction area of the scull by the scar. Top, one hundred days after operation; Bottom, 227 days after operation. M-muscle; S-scar; dura mater.

This is illustrated by the following experiment. Axolotl hind limbs are removed as close to the body as possible; the skin cuff is left as well as prepared *n. ischiaticus*. The removed part of the limb (skeleton, muscles, connective tissue, or muscles only) is minced and implanted into the skin cuff. Then *n. ischiaticus* is threaded through this mince and this sausage-like organ is left for grafting. If after complete grafting the end of this organ is removed, complete regeneration of the limb takes place (Fig. IV-3). If tail tissues are implanted into the skin cuff, the regeneration of a tail-like organ is observed. This clearly demonstrates that destroyed implant tissues represent a main source of the regeneration.

All this stimulated our attempts to obtain skull vault bone regeneration in dogs and other mammals using the destruction method. Initially experiments were performed with adult mice and rats and later with adult dogs. The results were hopeful, with dogs they were even better than with mice or rats. Therefore a brief account, given below, will be based on the results obtained with dogs.

When the pieces of parietal bone were removed in control animals the defect region was healed with fibrous tissue scars (Fig. IV-4). The edges of dense and hard bone in the defect region showed only a weak destruction. If the wound was covered by a specially prepared piece of pericranium, the latter fused with the dura mater by fibrous scar tissue.

In experimental animals a thick layer of freshly prepared bone dust (or particles) was implanted onto dura mater in the damaged region. These were prepared by tissue drilling with a manual surgical brace. The bone dust was wetted with recipient blood and antibiotic solutions. The upper surface of the transplant was covered (or was not covered) by a piece of pericranium, followed by the chewing muscle (m.masseter), galea aponeurotica and the skin. The animals were killed at different times during two years following the operation. Tissues from the region of the defect were studied histologically.

In all cases the regeneration of typical skull bones with bone marrow was observed, regardless of whether autotransplantation or homotransplantation was performed and whether bone dust was obtained from skull or tubular bones (Fig IV-5). With autotransplantation regeneration proceeded soli faster than in the case

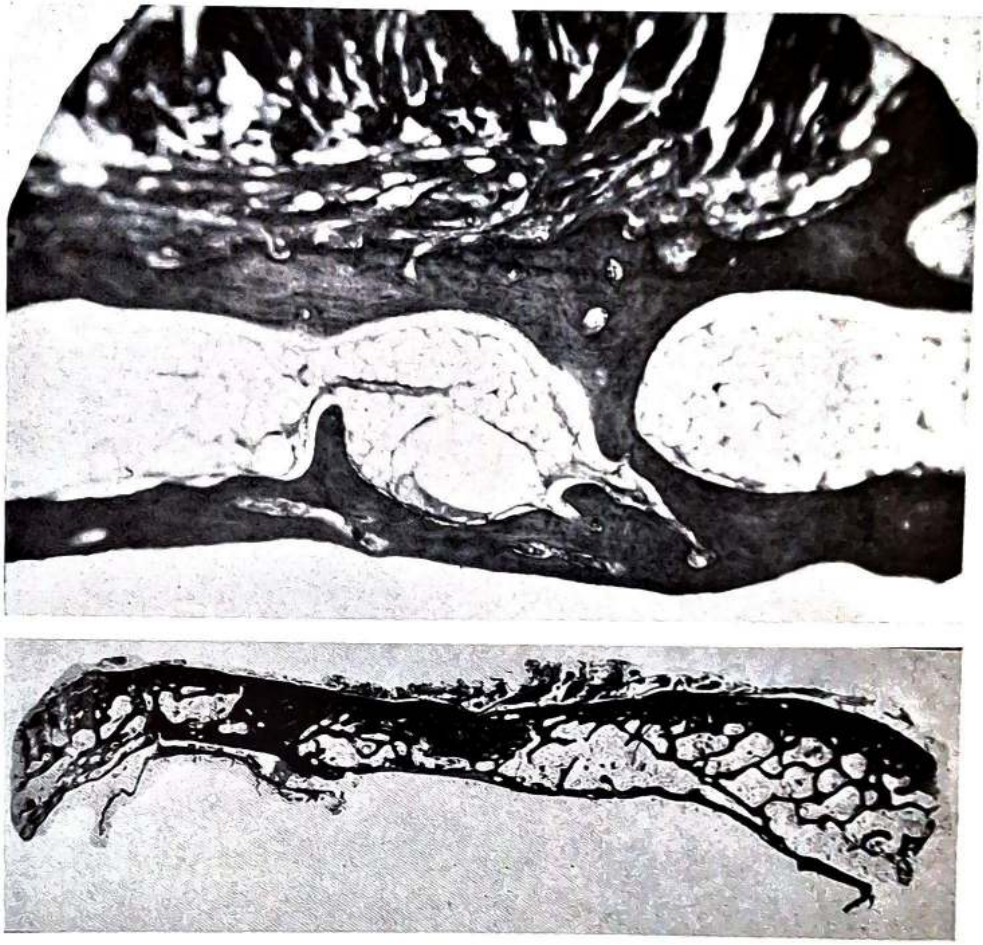

Figure IV-5. Regeneration of anatomically and histologically typical skull bone with bone marrow in dogs in destruction experiments. Top, 365 days after operation. Bottom, two years after operation.

of homotransplantation. Our latest results demonstrate that when bone crush prepared from cow bones is transplanted to the dog (e.g. with heterotransplantation) bone regeneration is observed (Kantorova, 1968a). Satisfactory results were obtained even with bone crush prepared from cow bones is transplanted to the dog at temperature 2—4°C. Lyophilization lowers while autoclaving for one hour at 120° completely abolishes the activity of such preparations. Besides bone crush, the presence of dura mater is a necessary prerequisite for the bone regeneration. If a piece of dura mater is cut from the wound region, no regeneration over this place is observed, although small islands of ossification are formed near the transplanted bone particles (Matveeva and Mitrofanova, 1963).

Histological studies have shown that bone dust serves as inductor of bone regeneration, not as a regeneration source or mechanical carcass for the bone formation.

The bone particles are of small size and they contain only a few osteocytes; these osteocytes do not survive more than one day after the operation. Bone particles are surrounded by a mass of erythrocytes, leukocytes and blood plasma (Fig. IV-6). In case of autotransplantation, bone particles completely dissolve during the first seven days after the operation by enzymatic action without the action of osteoclasts. This is accompanied by the substitution

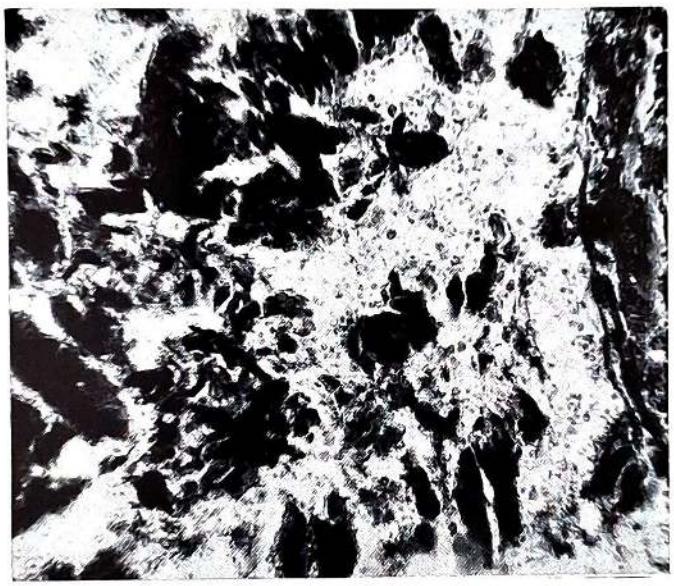

Figure IV-6. Bone dust and blood cells in the damaged area of the skull, three days after operation (Magnification: ocular 7, objective 8), (Matveeva, 1962).

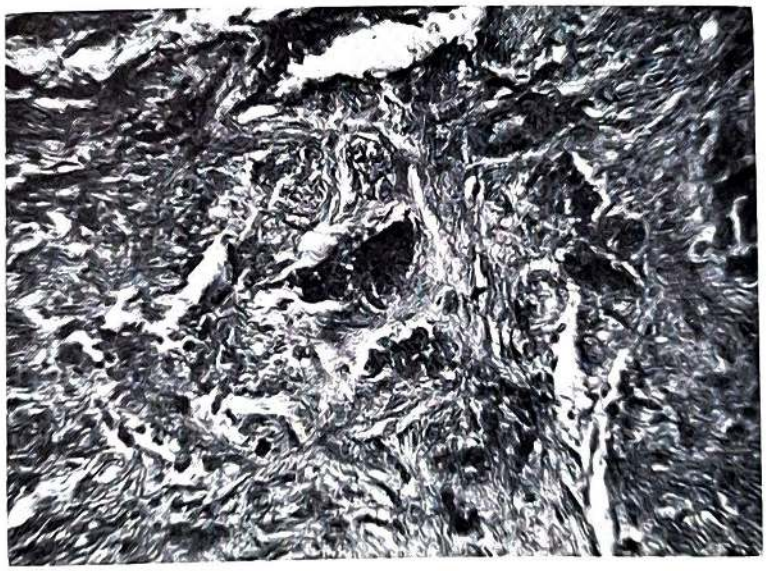

Figure IV-7. Damaged area of bone, five days after operation. Bone dust; connective tissue (Magnification: ocular 10, objective 20), (Matveeva, 1962).

of blood cells with the cells of immature connective tissue and a lot of new vessels are formed (Fig. IV-7). Substances liberated during the lysis of bone particles induce the formation of new bone from the immature connective tissue. This formation of the new bone begins near the edges of the wound and rapidly spreads through all the wound surface. It does not look like the wedge-like growth from the old bone to the wound center.

The formation of the new bone begins with the formation of aligned fibers near the dissolving bone particles. These fibers are interspersed with large embryo-like cells, containing large, light nuclei. These are induced osteoblasts. (Fig. IV-8). This is followed by the formation of irregularly shaped convoluted trabeculae of

Figure IV-8. Beginning of formation of bone beams in damage area of the dog skull, seven days after operation (Magnification: ocular 4, objective 90).

periosteal primary bone in the osteogenic tissue. Their outer surface is covered by a layer of osteoblasts while osteocytes are embedded in their inner ossified cavities (Fig. IV-9). Sometimes the remnants of incompletely dissolved bone particles are incorporated into these structures.

Ten to thirty days after the operation all the region of defect in the skull is filled by the young porous bone (Fig. IV-10). The trabeculae of periosteal primary bone become more thick and dense and are converted into the plates. Sinuses containing vessels, blood ductive regeneration has common features with embryonic induc-

Figure IV-9. Bone beams formed *de novo* in the damage area of the dog skull, seven days after operation (Magnification: 7 objective 40), (Matveeva, 1962).

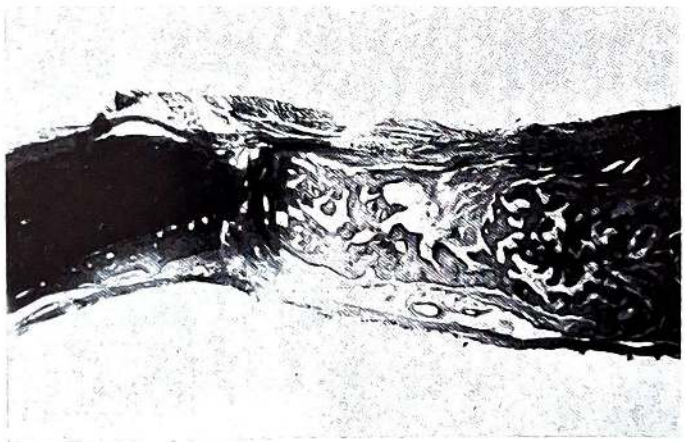

Figure IV-10. Cancellous bone filling the damage area of the skull in dog, fifteen days after operation (magnification: ocular 4, objective 42); old bone; periosteum; dura mater; regenerated bone.

cells and bone marrow are organized between these plates (Fig. IV-11). Then new bone grows and differentiates, fusing with the edges of the old bone.

Bone regeneration in the skull of adult dogs represents a special type of regeneration—regeneration by induction. It shows some characteristic differences from two other types of regeneration described in the literature—regeneration by morphallaxis that is rearrangement, epimorphosis or growth (Morgan, 1901). The inductive regeneration has common features with embryonic induction (Speman, 1936) and we can define such components or the

system as a) inductor—bone particles; b) reacting or competent material—cells of immature connective tissue; or c) a necessary condition for the induction process is the presence of dura mater. The process of induction involves the qualitative change of the reacting material under the effect of the inductor.

The process of tubular bone induction has been recently subjected to extensive investigation by autoradiographical, biochemical and histochemical methods (Urist *et al.*, 1967, 1968).

Regeneration by induction is quite well known in the case of the so-called Wolffian lens regeneration from the dorsal edge of the iris in the adult newt (Colucci, 1891; Wolff, 1895). Tissue induction in the organism of adult rabbits was demonstrated by Levander (1964) with his originally developed method of tissue treatment by trypan blue solution.

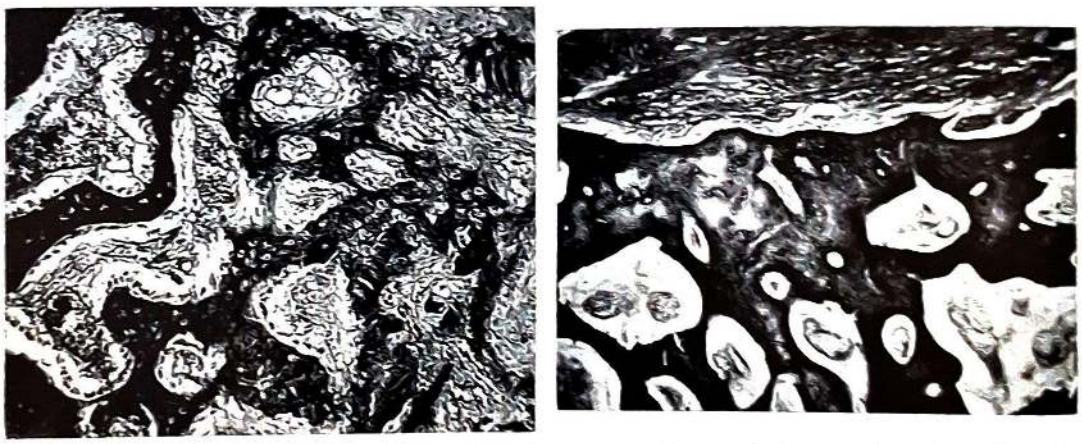

Figure IV-11. Bone regenerated after operation of the calvarium in dog (Matveeva, 1962). Left, After ten days; magnification: ocular 10, objective 8; Right, After fifty-nine days; magnification: 7, objective 8.

CONCLUSION

The recovery of lost ability of animal organs to differentiation demands strong stimulation of destruction and dedifferentiation processes in tissues of these organs. On the basis of this concept, regeneration of skull vault bones in mice, rats, dogs and some other mammalian species can be obtained. The transplants used in these methods serve as *inductors* of regeneration, they do not yield material for the substitution of the lost bone. It follows that homotransplantation and heterotransplantation can be performed, since tissue incompatibility of donor and recipient needs not be circumvented.

The destruction method is most simple of all four for the biological substitution of skull bone defects. It gives 100 per cent regenera-

tion of vault bones, which do not regenerate under normal conditions. The defects may vary in size and in shape. These results were confirmed not only with dogs (Kovalevsky, 1967, 1968), but also with human patients (surgeons Strebkov, 1966; Volkov, 1968).

The method of bone destruction was used in surgery but in a different way. To increase the surface of bone fragments Kartashev (1930) crushed tubular bones into pieces 5 to 10 mm long. After the transplantation, this broken bone fused and bone regeneration was observed. Experiments with crushed bone and bone chips for the stimulation of tubular and maxillary bone regeneration were conducted by several surgeons (Sitenko, 1935; Moulem, 1944; Bolotin, 1945; Gordon, 1946; Rusanov, 1958; Olshvang, 1956; Lavrishcheva, 1955, 1957; Balakina, 1956; Zakharzhevsky, 1958).

When broken bone fragments were implanted into the damaged region in the human skull, an x-ray image has shown that regeneration of bone tissues could be detected one to two years after the operation (Izvekova, 1959). These results demonstrate practical applicability of the destruction method. It should be remembered however that it was not demonstrated in these cases that the bone regeneration occurs through the induction. In a number of cases the transplants simply survived and filled the regions of bone defects.

In cases described by the author regeneration occurs *via induction*. Strong destruction of the bone leads to its regeneration. This is accompanied by the dissolution of the bone particles and liberation of inducing osteogenic substances. The nature of these substances is not yet known. It may be that they represent proteins or RNA. It has been recently demonstrated that organ-specific RNA, isolated from tubular bones, stimulates tubular bone regeneration in rats (Belous, 1968, 1968a, 1970). The bone particles themselves are not dedifferentiated. But they affect the surrounding cells of immature connective tissue and lead to their dedifferentiation and qualitative change, which finally results in the formation of bone tissue with bone marrow (Büring and Urist, 1967).

The demonstration of a new type of regeneration, regeneration by induction, in addition to older known types, may have considerable theoretical and practical significance.

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V

INDUCTION OF TOOTH TISSUE REGENERATION

It has been shown in the preceding chapter that regeneration of skull vault bones, which do not regenerate under usual conditions, can be achieved by relatively simple means. The skull bones are characterized by their high density and hardness; when bone fragments are removed, the edges are not destroyed and not dedifferentiated. If bone destruction is stimulated artificially, the onset of regeneration is observed. Therefore a question may be asked, whether tooth tissue, the most dense and hard tissue in the organism of mamals and man can be evoked to regeneration.

It is well known, that the channels drilled by dentists bore in the tooth crown are never filled with the regenerating tissue. These holes are usually filled with phosphate cement or other suitable substances. Can the healing of damaged teeth be achieved by induction of tooth tissue regeneration?

The inability of tooth tissues to regeneration may depend on one of two factors: a) the treatment of teeth during the medical procedure by chemical agents which kill living tissues capable of regeneration and b) the absence of biological conditions, favorable for the regeneration process. To solve the problem, therefore, drugs, inactivating living tissue, should not be used and biological conditions facilitating regeneration are to be found. From the standpoint of biochemical composition and histological structure, tooth tissues—dentin and cement in particular—are very similar to the bone tissue. Therefore there are reasons to believe that under certain conditions tooth tissue may also be induced to regeneration (Polezhaev, 1961; Polezhaev, Matveeva and Vorobieva, 1958).

Adult dogs two to five years old were used in the experiment. Fangs of upper and lower jaws were chosen as experimental models; operations were performed on animals narcotized by

morphine-ether under aseptic conditions. The tooth was thoroughly sterilized by 70°C ethanol. After the operation, animals received three daily injections of penicillin, 100.000 units each. The animals were sacrificed at different times up to 436 days after the operation. Histological sections or polished preparations were prepared from the teeth. For that purpose they were cut at the neck level and fixed in 20% formaldehyde. The plane of the section or the polished surface in case of grounds crossed the tooth channel and cavity drilled by the bore. Bore number 3 was used; the sections were stained with hematoxylin-eosine.

TOOTH TISSUE REGENERATION INDUCED BY DESTRUCTION METHOD

It has been already noted, that tissue destruction may lead to

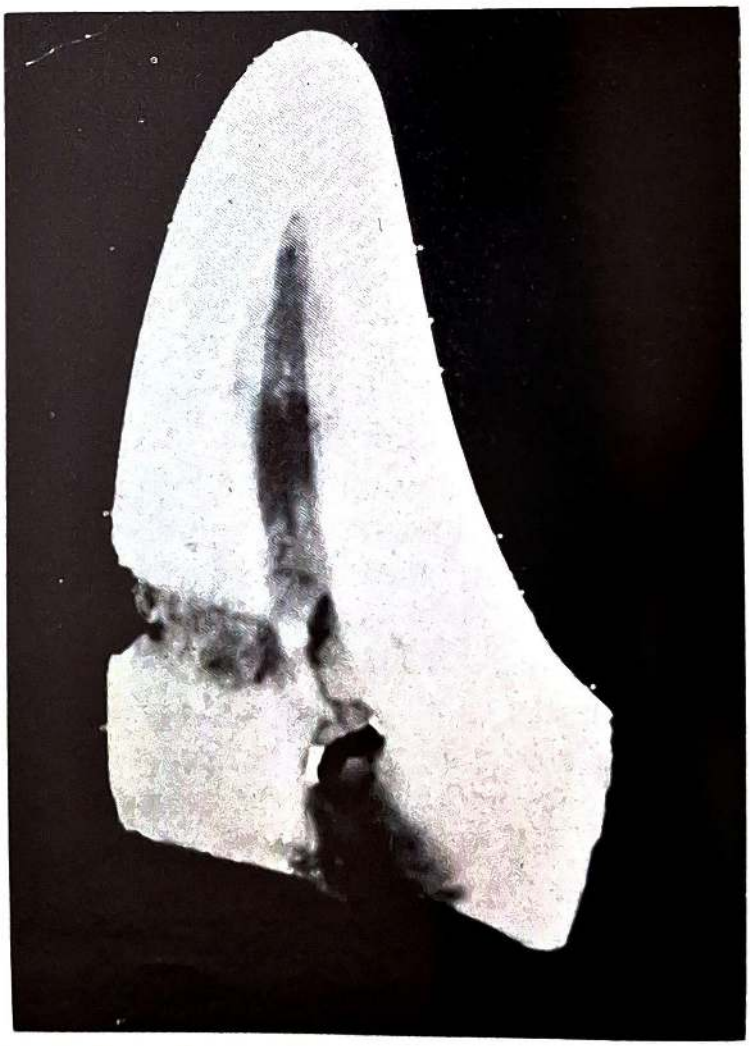

Figure V-1. Section of a dog fang one hundred days after operation. The tooth chamber is closed by dentin-like tissue formed *de novo*.

limb regeneration in axolotles (Polezhaev, 1934, 1936) or to regeneration of skull vault bones in mammals (Polezhaev, 1957; Matveeva, 1962; Kantorova, 1968). Studitsky (1959), using the destruction method, obtained skeletal muscle regeneration in mammals. His results were confirmed by Carlson (Carlson, 1969, 1970, 1970a). In experiments with tooth regeneration in dog's, holes were bored in fangs until they met the tooth channel.

The hole diameter was 3-4 mm, its depth attained 5-8 mm. Dentin particles were collected into a rubber cup, put onto the tooth crown during the drilling. The dentin particles so obtained were slightly wetted with penicillin solution, prepared on the 0.85% physiological saline, the resulting paste was used for filling of the bored chamber. The uppermost part of the chamber was filled with a phosphate-cement layer 1-1.5 mm thick; it was used

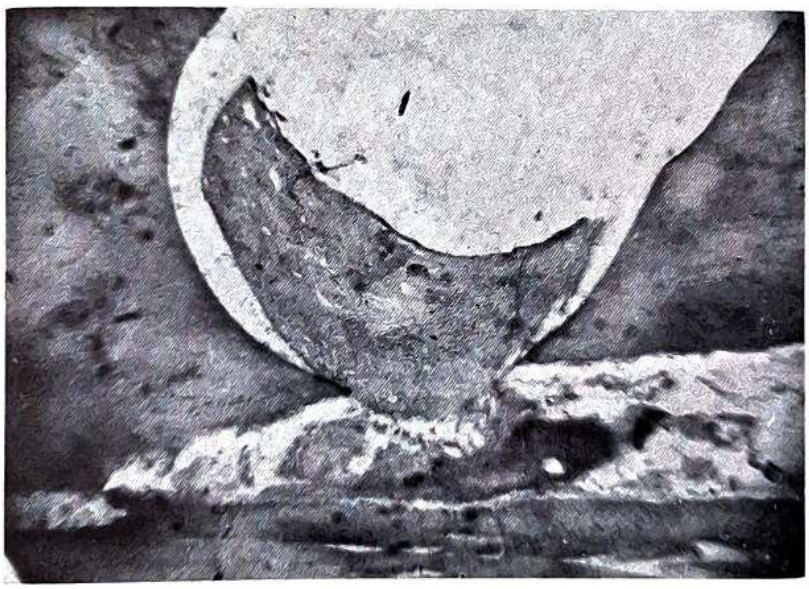

Figure V-2. Operation area in the tooth section. Dentin-like implantant.

for mechanical retention of the implanted dentin particles. The phosphate-cement filling fell off at different times, usually four to eight weeks after the operation, sometimes it remained in the tooth as long as one year.

In all experiments the result was very definitive—biological implant was converted into a bone or dentin-like tissue which completely plugged the tooth chamber (Fig. V-1). This was accompanied by obliteration of the middle part of the dental channel which was in contact with the chamber containing the implant. In

this region the pulp was converted into the osteoid tissue and a layer of new dentin was formed on the dentin inner surface (Fig. V-2). In the bored tooth wall dentin was only slightly affected. It consisted of basic substance with typical radial, somewhat convoluted channels, penetrated with odontoblasts processes (Fig. V-3).

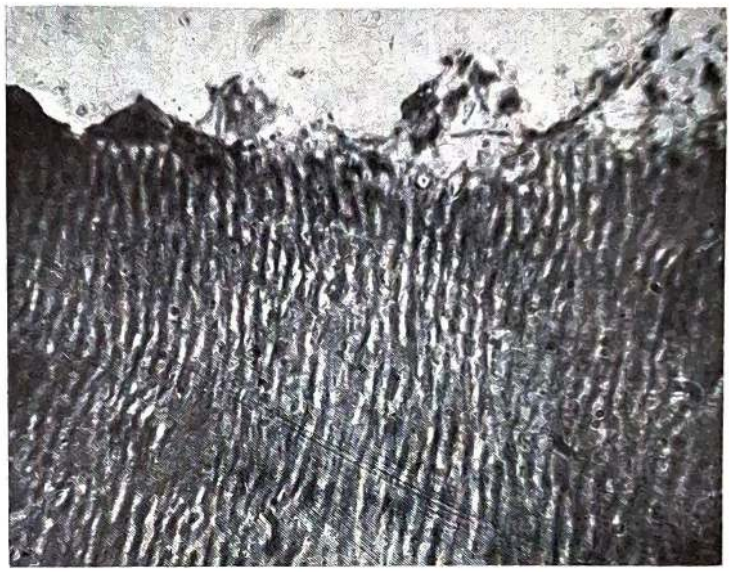

Figure V-3. Canalized dentin in the tooth wall and odontoblasts (magnification: ocular 10, objective 60).

Its inner surface was in contact with odontoblasts and soft from connective tissue pulp. A thick layer of new, induced dentin was formed on the inner sides of the dental channel, just opposite the implant (Fig. V-4).

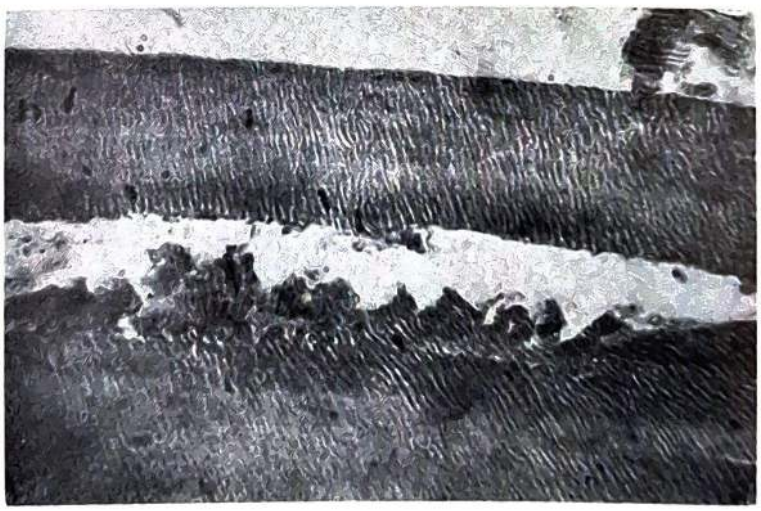

Figure V-4. Dentin formed *de novo* and scaled off the tooth wall (magnification: ocular 10, objective 60).

Implant, placed into the tooth chamber, forms a plug, which consists of very small dentin particles firmly glued to each other by some amorphous substance. This tissue contains neither cells nor their processes. Gradually this tissue becomes more and more dense; forty-five to sixty days after the operation the glued dentin particles retain their initial structure—dentin channels are seen relatively clearly. Dentin particles are glued to each other in a relatively soft manner. Later, one hundred days after the operation, the structure of particles undergoes certain changes, dentin channels are very clearly seen, the amount of cementing substance increases and the plug structure becomes more amorphous and dense (Fig. V-5). It can be concluded that implant tissue develops, and

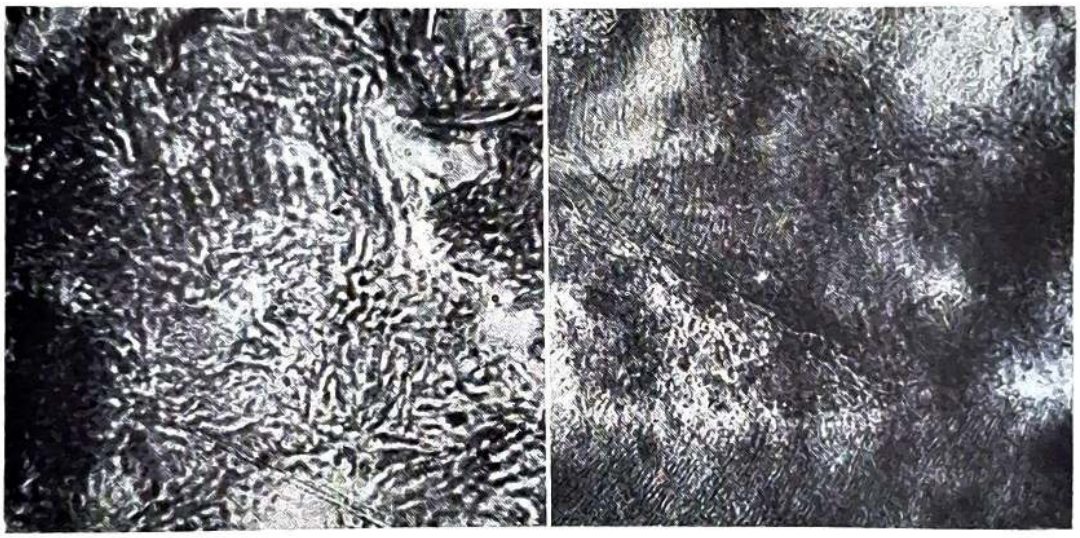

Figure V-5. Structure of dentin-like tissue in the tooth chamber (ocular 10, objective 60). Left, Looser tissue; Right, Denser tissue.

metabolic processes occur within it. The bone-like plug, formed from the implant, is a safe protection of the tooth from infection. Even one year after the operation after the slipping out of the phosphate-cement filling the plug serves as a very efficient protection for the chamber and the pulp—no caries or pulpite was detected. The implant tightly fits to the walls of the dentin chamber, but these do not regenerate.

All of the results show that dental tissue which is unable to regenerate under usual conditions can be stimulated to regeneration and metaplasia in our experimental conditions (strong destruction).

When this work was finished, we learned from the literature that

there were some achievements in this direction in the field of practical stomatology. Feldman (1932), for example, suggested a new approach to the therapy of teeth with pulp inflammation. He came to the conclusion that uses of drugs resulted in the necrosis of pulp stump and scar formation. When drugs were not used, pulp regeneration could be evoked under certain biological conditions. He bored a chamber in a healthy dog tooth, removed the pulp and covered the stump with dentin particles. In all cases the stump remained and was converted by metaplasia into a dense osteoid tissue with incorporated aggregates of dentin particles covering the bottom of the chamber. A layer of new dentin was formed around the implant and the wound was healed. If the chamber, however, was treated with phenol or cobalt, then in the absence of dentin particles pulp degenerated and finally perished. This author successfully used his aseptico-biological method for two years. Inflammation and pulp stagnative degradation was healed in this way when he removed the inflamed pulp and covered it with a layer of dentin particles.

Our results, presented above, are in full agreement with the data reported by Feldman (1932). He, however, was interested in the pulp, while we dealt with dentin regeneration. Feldman has shown that pulp can survive when its metaplasia into the osteoid tissue takes place. This occurs under the effect of dentin particles. Our results confirm his observations and in addition they demonstrate the possibility of atypical dentin regeneration from dentin particles; this leads to the possibility of biological teeth filling. It is known from the general principles of regeneration that regeneration of a certain particular organ stimulates regeneration and development of another organ. This means that induction of teeth tissue regeneration in case of caries, for example, may lead not only to the healing of caries but also to the strengthening and sanitation of other teeth. Dentin regeneration by the destruction method represents a new approach to teeth biological filling. It has the following advantages: a) it permits the survival of tooth tissues in the living state; b) it stimulates new growth and development in the pulp and in dentin tissue; and c) it may lead to general strengthening of teeth adjacent to those which underwent such healing.

It should be noted that much remains to be done for the prac-

tical development of this method—experiments with homoplastic transplantation of dentin particles must be conducted; lyophilized and frozen dentin particles must be tried; the possibility of enamel regeneration induction must be investigated and so on.

TOOTH TISSUE REGENERATION EVOKED BY INDUCTION METHOD

Regeneration of dentin and cement of tooth root has been demonstrated in dogs by Gavrilov (1957). The root was uncovered by alveolotomy; a hole was bored and this region was closed by a piece of mucosa and periosteum. After ten days the region of the defect was filled with fibroblasts, after fifteen days homogeneous predentin was formed, after thirty days dentin was filled with canaliculi and cement, after ninety days dense dentin and cement were observed in the affected region. This demonstrated the possibility of genuine tooth tissue regeneration which occurred by conversion of young undifferentiated connective tissue, filling the damaged zone. Such experiments, however, may be conducted only with tooth root, not with its crown located over the amphodont. But healing and filling performed by stomatologists usually are made with the crowns damaged by caries.

We have made an attempt to evoke tissue regeneration in the tooth crown, using a piece of differentiated connective tissue placed into the damaged hole together with dentin particles. The connective tissue served as reacting material, while dentin particles served as an inductor. In other words, we wanted to develop a method of teeth healing by biological filling based on metaplasia and tissue induction.

For this purpose a hole was bored (bore number 3) in dog fangs, this hole reached the dental channel. Two or three pieces of amphodont or galea aponeurotica were transplanted into the bored chamber, they filled it practically to the surface. A part of the implant touched the pulp of the dental channel. From the outside the implant was covered with phosphate-cement filling, which protected it from infection and slipping out. Amphodont connective tissue was taken from the gum which was thoroughly washed by 70% ethyl alcohol, then the external epidermis was cut. A piece of galea aponeurotica was taken from the head of a dog subjected to the operation of the skull. Small amounts of dentin

particles obtained during the tooth boring were implanted together with amphodont pieces or galea aponeurotica. These dentin particles might lead to induction and consequently to metaplasia of the implanted tissue. The implant was slightly wetted by penicillin solution prepared on the physiological saline. The animals were killed at different times, up to one hundred days after the operation. The phosphate-cement filling remained in its place in all nine cases, except one. All animals felt well after the operation, no symtoms of tooth pain was recorded. Procedure for histological preparations was as described in the above experiments.

The results were definitive and unequivocal—in all nine cases biological implant was converted into the dense, hard bone-like

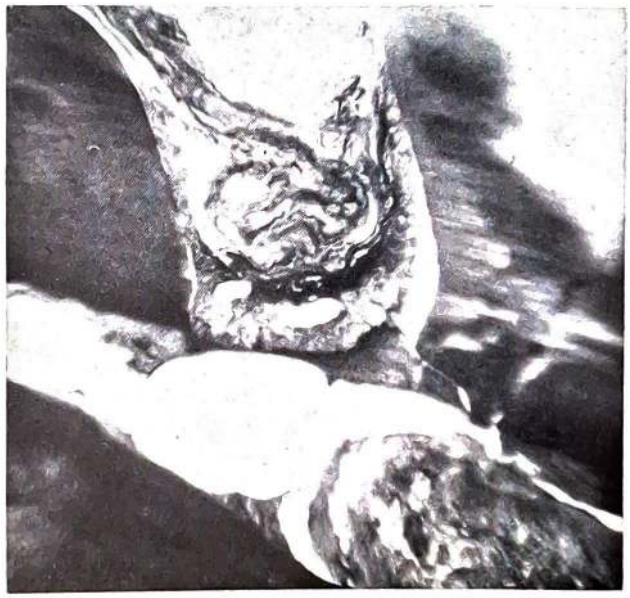

Figure V-6. The dog tooth chamber closed by dentin-like tissue. The tooth canal contains induced osteoid tissue.

tissue, which tightly plugged the chamber in the tooth (Fig. V-6). The amphodont converted into the genuine bone or dentin, while galea aponeurotica converted into the mineralized dense tissue which differed from the bone by its structure. The pulp of the dental channel under the influence of the implant was converted into the dense osteoid tissue. The formation of new dentin took place on the inner tooth wall.

There are two stages in the conversion of the implanted tissues. At the first stage, during fifteen days after the operation, there is only incomplete conversion into the bone or osteoid tissue; at the

second stage between thirty and one hundred days after the operation there is complete and deep conversion of the implant into the osteoid or dentin-like tissue.

The conversion of the implanted amphodont begins seven days after the operation, it proceeds irregularly in different regions. The

Figure V-7. Implanted amphodont. Seven days after operation. It consists of fibroblasts and thick dense collagen fibers (ocular 10, objective 60).

Figure V-8. Transformation of amphodont to bone in the tooth chamber, seven days after operation (ocular 10, objective 60).

amphodont piece, located in the dental channel, retains its initial histological structure and is not converted into the bone (Fig. V-7). It consists of relatively large, rare, separately located fibroblasts with distinct nuclei, nucleoli and spindle-shaped cytoplasm. The fibroblasts are interspersed with multiple thick convoluted collagen fibers, which do not show any signs of mineralization or conversion into the bone. Situation is quite different in the amphodont piece placed into the bored chamber. This piece is converted into bone. Amphodont tissue contains relatively many dentin particles, and near the surface covered with phosphate-cement filling, it consists of compact bone layers, which show staining different from that of undifferentiated amphodont (Fig. V-8). The shape of fibroblasts is also altered. They are long, narrow and embedded into the basic bone substance. Initially fibrous bone is formed, and later it is converted into the compact one. At even later times, for example, forty-three days after the operation, very dense bone with light color is formed in the amphodont of the chamber; here rare osteocytes are embedded into the basic substance (Fig. V-9). Amphodont pieces located in the pulp of the dental channel are also converted into bone, but much more slowly than those in the

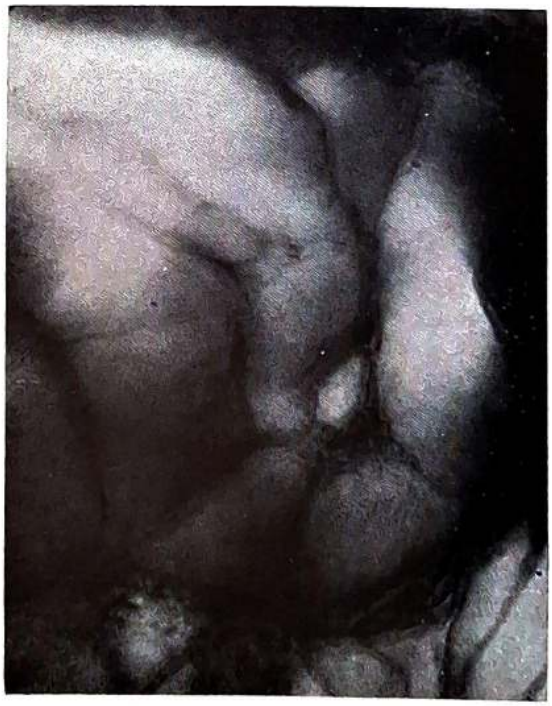

Figure V-9. Further transformation of amphodont, which was implanted into the tooth chamber, to dentin-like tissue with immured osteocytes. Forty-three days after operation (ocular 10, objective 60).

chamber. The structure of this bone is also different from the structure of bone developed from the amphodont in the chamber. This is a dense mineralized compact bone; it shows irregular structure and consists of the changed amphodont, pulp and dentin particles fused together. It plugs the opening of the dental channel.

The development of galea aponeurotica, transplanted into the chamber of the tooth crown and into the dental channel occurs in quite another way. Normally galea aponeurotica consists of loose jelly-like connective tissue, containing a few rare fibroblasts and abundant intercellular substance. After the transplantation into the chamber, this tissue, containing many dentin particles, becomes mineralized and dense. It looks like osteoid tissue, but does not differentiate and is not converted into bone (Fig. V-10).

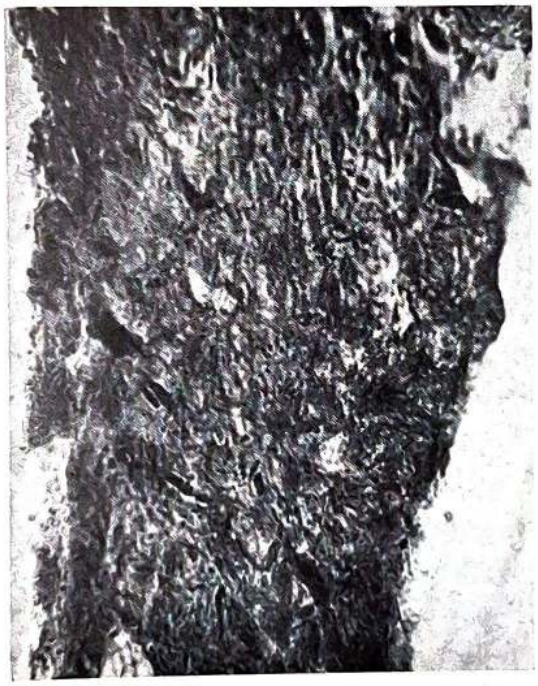

Figure V-10. Aponeurosis grafted into the tooth chamber. It is petrified but does not transform into bone. Thirty days after operation (ocular 10, objective 40).

Considerable changes take place in the pulp. Immediately after the implantation the aseptic inflammatory process begins, and pulp becomes heavily infiltrated with granulocytes and lymphocytes. Sometimes this can last as long as ten to fifteen days. This inflammation is accompanied by the formation of large cavities, filled with some fluid. On the other hand, maturation of pulp connective tissue is observed, and fibroblasts become aligned in dense rows on

the walls of the dental channel.

Thus connective tissue of different origin shows different behavior when it is placed under the same conditions—amphodont tissue undergoes conversion into bone, while galea aponeurotica does not, although both of them are placed into the tooth chamber together with dentin particles. It can be inferred that morphogenetic potencies of connective tissues of different origin are different.

The conversion of transplanted amphodont into bone without preceding proliferation of its constituent cells and without the formation of new connective tissue is an example of direct metaplasia. Similar direct metaplasia of dense fibrous tissue into bone was described by Sipovsky (1961).

In our experiments the conversion of transplanted connective tissue into the dentin-like one might depend upon the influences of dentin particles or possibly of phosphate-cement filling. This osteogenic influence was inductive in its nature, dentin particles and dentin of the tooth chamber served as inductors. Amphodont tissue represented the reacting system. The amphodont origin was the intrinsic condition of the induction process.

The conversion of amphodont tissue into the bone or dentin-like structure after the transplantation into the tooth chamber represents an induction process, similar to embryonic induction, for example, the conversion of newt early gastrula ectoderm into the nervous tissue. This conversion is induced by invaginating presumptive chordomesoderm (Spemann, 1936). A similar example is the conversion of embryonic mesenchyme into the cartilage capsule of the ear vesicle (Filatov, 1939). These are examples of embryonic tissues which are pluripotent. In our case the conversion of differentiated tissue of adult animals is observed. These data are similar to the results of Levander (1964) on tissue induction in adult rabbits.

Both our experiments and experiments of Levander concern tissue induction in adult mammals. The critical difference is that in his experiments the tissue is induced at the improper place and then rapidly resolves. In our experiments we induce regeneration of the tissue, or a part of the organ, which is not regenerated under normal conditions. The induced tissue is stable and survives for a long time. The results obtained with the regeneration of tooth tissues and skull vault bones are similar. Both organs are incapable

of regeneration under the conditions of usual damage, both cases refer to mammals and the types of regeneration are also very similar. The principal difference is the fact that in experiments with skull bones the newly formed part of the organ originates from the immature connective tissue, while in experiments with teeth it arises from the mature, differentiated fibrous tissue. This probably explains the difference between the pattern of regeneration with skull bones and with teeth. In cases of skull bones the regeneration results in the formation of typical skull bone, as shown both by anatomical and histological criteria in the case of tooth regeneration somewhat atypical tissue is formed—this tissue does not contain dentin canaliculi. The regeneration of skull bones involves both organogenesis and histogenesis, tooth regeneration involves histogenesis only. It can be suggested that the use of young fibroblasts for tooth tissue regeneration might lead to the formation of dentin canaliculi as well.

SUMMARY

(1) The autotransplantation of amphodont mixed with dentin particles into the chamber bored in the tooth of the adult dog results in the conversion of the latter into either bone or dentin by direct metaplasia. Under the same conditions the transplanted galea aponeurotica becomes dense and mineralized but is not converted into bone or dentin. The conversion of the connective tissue into bone under these conditions is inductive in nature, tooth tissue regenerates by induction.

Both methods of dental tissues regenerate by induction and by destruction, demand the abstention from the drugs used in stomatology, which damage and destroy living tissue. On the contrary, biological conditions must be found which lead to the regeneration of tooth tissues which normally are incapable of undergoing such regeneration. We think that the results obtained by us have not only theoretical but also practical significance. At present there are some attempts to apply certain principles of biology in tooth healing. At first, a number of authors tried to transplant a new living tooth on the place of the old absent one. In this area probably the attempt to transplant tooth rudiments to adult dogs and rats are most promising (Lapchinsky and Malinovsky, 1940; 1940a; Lapchinsky, 1941). It was demonstrated that teeth rudiments

transplanted into the soft tissues cannot develop and die, while when transplanted into the femoral or jaw bone they are capable of growth and differentiation. Then there are attempts to achieve the alloplastic teeth substitution. In this approach perforated metal or plastic artificial teeth are grafted into the gum. The perforations become filled with connective tissue, which secures these teeth on their place (Vares, 1957 etc). The approach described above may serve as the third biological principle of teeth healing. It is based on the principles and phenomena of regeneration. This attempt consists of special treatment upon the tissue, so that its state is changed, inductive and metaplastic processes are evoked with the consequent recovery of tooth tissue ability to regeneration. In contrast to the other method, this approach can be used not for the substitution of the absent teeth, but for the healing of the damaged ones. Its proper development may lead to certain positive results.

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VI

THE RECOVERY OF HEART MUSCLE

FORMULATION OF THE PROBLEM

The recovery of vitally important organs, which are unable to regenerate under normal conditions, appears most interesting from both theoretical and practical viewpoints. Heart and brain are the examples of such organs. Cardiovascular diseases are responsible for the large proportion of lethality in industrial countries. The heart muscle is most frequently damaged. This damage includes myocardial infarction, myocarditis of various types, predominantly of infectious origin (Diphtheria, scarlet fever, influenza, rheumatic diseases, etc.), myocardiodystrophy, wounds and so on. At the present time a number of heart diseases can be treated surgically. This also demands the intensification of studies on stimulation of wound healing or heart muscle regeneration.

Presently heart diseases are treated by placing the patient under strict resting conditions and using a variety of chemical drugs for improvement of the cardiac function. Under these conditions the resolution of necrotic lesion in the damaged tissue takes place, which is followed by its substitution with the scar fibrous tissue. The damaged parts of the heart, however, are not regenerated. The scar formation results in hypertrophic growth of the remaining part of the heart muscle and leads to a certain compensation of the heart activity.

Therefore the recovery of damaged heart muscle in mammals under experimental conditions is a very attractive goal. We made an attempt to approach this problem from the viewpoint of the regeneration principles outlined in the first chapter, that is to induce heart regeneration by augmenting destruction and dedifferentiation of tissues in the damaged heart regions. Experiments were conducted with adult rats, rabbits and dogs. Methods and results of this work, which lasted for several years were summarized in a

number of papers and in a monograph (Polezhaev, Akhabadze, Muzlaeva and Javich, 1965). Only the most important results will be presented below.

A reservation should be made from the very beginning, that the problem of heart muscle regeneration was not solved by us, however a certain path to its solution was laid down. Certain positive results were obtained; some of them were confirmed in a number of laboratories, but disputed by other investigators.

DATA ABOUT THE INABILITY OF HEART MUSCLE TO REGENERATE

There is an abundance of experimental data concerning the reparative regeneration of heart muscle in adult rats, rabbits, cats, guinea pigs, hamsters and dogs (for literature see reviews Marchand, 1901; Goldzieher and Makai, 1913; Mönckeberg, 1924; King, 1941; Polezhaev and co-workers, 1965 etc.). It has been shown that damage of the heart muscle caused by cut and stab wounds, burns, introduction of steel or gelatin needles, by ligating the left descending branch of the coronary arterium leading to infarcture, does not lead to regeneration of heart muscle fibers. Similarly, regeneration does not take place when heart muscle is damaged by resection of its part, by experimental myocarditis, by the action of nutrition or physical factors or by stress. Usually the damaged region becomes enveloped with inflammation, with subsequent formation of granulation tissue and scarring. Similar phenomena are observed with human patients in various cases of heart muscle damage, such as wounds, infarctures, myocarditis and so on.

Some authors were able to record only fragmentary regenerative phenomena such as amitotic and, only very rarely, mitotic division of nuclei in muscle fibers stumps, or the migration of myogenic cells into the damaged zone. These myogenic cells separated from the destroyed muscular stumps and mixed with the cells of connective tissues ("myogenic granulations," described by Oppel, 1901, Anichkov, 1913, 1913a).

The inability of heart muscle to regenerate is explained in different ways by different authors. These explanations are as follows: insignificant development of sarcolemma in heart muscle fibers; rapid growth of connective tissue, which prevents the regeneration of muscle fibers; the decrease of the ability for nuclear division with

age. Investigations, conducted during the last few years using methods of autoradiography and cytophotometry, have shown that the ability to DNA synthesis and division of muscle nuclei is lost at the early stage of the postnatal ontogenesis and is very low in adult animals (Macdonald and Mallory, 1959: Messieur and Leblond, 1960; Shorter and Titus, 1962; Pelc, 1963, 1964; Wegener, Hollweg and Maurer, 1964; Rumyantsev, 1963, 1966; Peterson and Baserga, 1965; Overy and Priest, 1966; Klinge and Stöcker, 1968). These results lead to the opinion that inability of heart muscle fibers to regenerate is due to steady repression of DNA synthesis and mitotic divisions in them (Mirakian and Rumyantsev, 1968).

Klinge (1967) has established in his radioautographic investigation that stimulation of DNA synthesis and nuclear division is observed in muscle fiber stumps after the experimentally induced myocardial infarctions. It should be noted that mitoses were anomalous, with the impairment of achromatic apparatus of the mitotic spindle. Mirakyan and Rumyantsev, however, dispute this conclusion in their paper cited above. At any rate, even if there is slight stimulation of DNA synthesis in muscle stump nuclei, the fibers are unable to regenerate under the conditions of usual damage.

DATA SHOWING THE ABILITY OF HEART MUSCLE TO REGENERATE

It was shown that in newborn rats or kittens muscle fibers could regenerate when the wall of the left ventricle of the heart had been perforated with the needle or had been burned (Rumyantsev, 1954, 1955; Robledo, 1956; Mirakyan and Rumyantsev, 1968; Akhabadze and Nymmik, 1967). It was described that heart muscle fibers damaged by diphtheria myocarditis in human infants could regenerate (Heller, 1914; Warthin, 1924). Regeneration of heart muscle fibers was demonstrated in adult frogs and lizards (Kolosova, 1961; Rumyantsev, 1960; Sulima, 1968, 1969).

In early experiments with the explants of heart pieces, dedifferentiation of heart muscle, growth of fibroblasts and pulsation of the explant was recorded (Rumyantsev, 1932; Murray, 1965). Only during the last few years secondary differentiation in explants of chick or human embryonic heart was demonstrated (Kochetov,

1961; Agrell, 1965). Under special experimental conditions (in the medium containing embryonic extract only) good secondary differentiation of heart muscle fibers from the adult cock has been observed (Grigoriev, 1957; 1960, 1964) (Fig. VI-1,2).

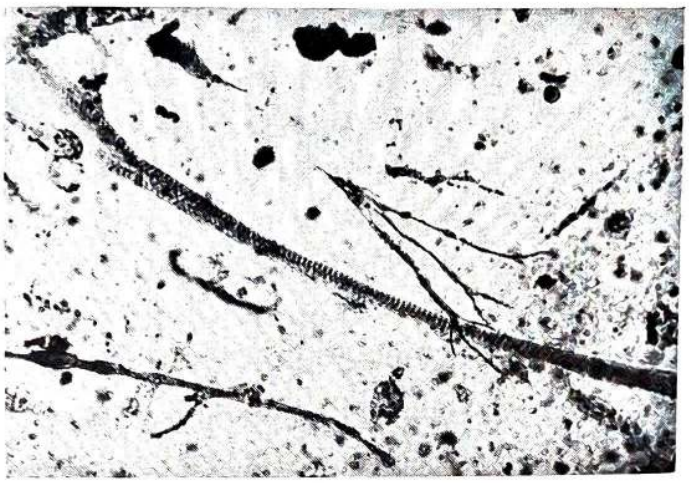

Figure VI-1. Secondary differentiation of muscle fibers of explanted cardiac muscle of twelve-day-old chick embryo. Culturing for ten days. General view. Bielschowsky-Boeke. (Grigorev, 1960).

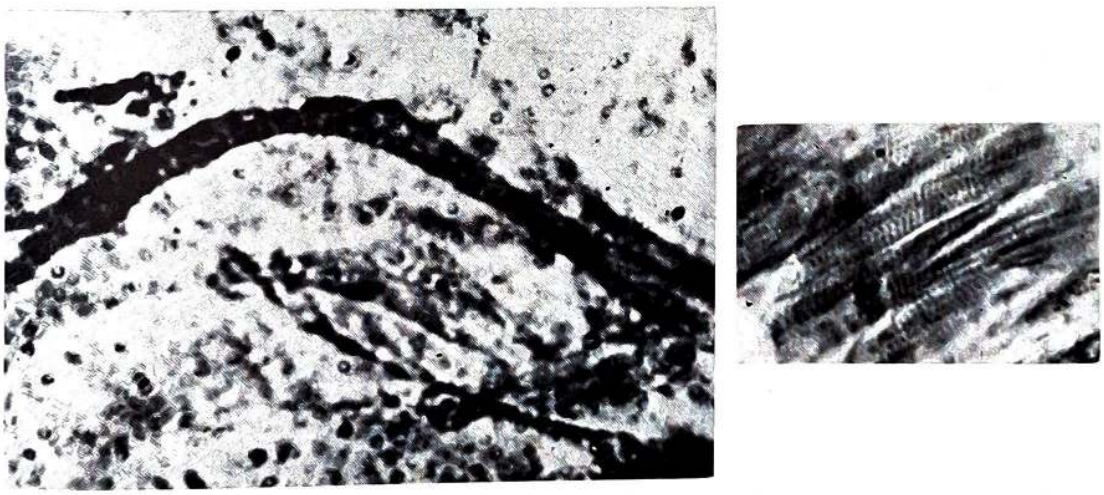

Figure VI-2. Secondary differentiation of muscle fibers of myocardium of adult two-year-old cock *in vitro* (Grigorev, 1964), Bielschowsky-Boeke. Left, Secondary differentiation of muscle fibers in the growth zone. Ocular 10, objective 40; Right, Mitosis in muscle fibers of myocardium cultured for twelve days, Ocular 10, objective 40.

Törö (1939) has demonstrated that extracts from the embryonic heart can stimulate growth of chick embryo heart pieces cultured *in vitro*. However this extract failed to stimulate muscle fiber regeneration after the infarction in adult rats. Nevertheless these experiments paved the way for the investigations, which showed that

protein-free extracts from cattle heart muscle (recosen) may stimulate heart function both in animals and in human patients (see Polezhaev, *et al.*, 1965).

OUR APPROACH TO THE PROBLEM OF HEART MUSCLE REGENERATION

It is clear that mammalian heart muscle is capable of regeneration at early stages of postnatal ontogenic devolpment, while later this ability is lost. A similar pattern of changes in regeneration ability was observed for the limbs of tailless amphibia (Anurans) and for the skull vault bones of rats, dogs and humans. Thus there are some grounds for the attempts to recover the lost ability of heart muscle to regenerate, as was the case with limbs and skull vault bones.

According to our concept, the recovery of ability to regenerate demands drastic stimulation of destruction and dedifferentiation in the damaged part of the organ. The surgical method of such stimulation is, of course, inapplicable to the heart muscle. Therefore in order to stimulate regeneration we decided to apply physiological means, that is to administer heart muscle degradation products to the animal with the damaged heart muscle. This approach follows from a number of considerations. Tissue degradation in the organ can be augmented by the administration of degradation products from certain tissues. Nassonov (1941) implanted pieces of cartilage under the skin of axolotl limbs. The cartilage was degraded, destroyed the surrounding tissues and induced the formation of additional extremities. Zelinsky (1946) observed similar effect, when he administered cartilage hydrolysate under the skin of the extremity. Polezhaev and Ramenskaya (1950) induced regeneration of extremities in adult *B. bombina* by the administration of cartilage hydrolysate under the epithelium of the wound surface after limb removal. It should be remembered that extremities in this species are incapable of regeneration under the usual conditions. It follows from the principle of organ specificity (see Chap. I) that myocard must be most strongly affected by the degradation products of the myocardium itself. Therefore we decided to prepare products of heart muscle degradation and to inject them into the animals with the damaged heart muscle.

It is known from the literature that hydrolysates and lysates of

tissues were successfully used by Tushnov (1931, 1938) for the stimulation of impaired organ functions. Tissue autolysate was employed for the skin wound healing (Selkov and Vasiliev, 1935); Sozon-Jaroshevich and Goldberg, 1936 etc.). Tissue therapy employed by Filatov (1945) and Rumyantsev (1951, 1953) also leads to the increase of the regeneration ability. Healing with lyophilized tissues was employed by Niehans (1948, 1955, 1964) and Kuhn (1956). Recently, preparations of organ specific RNA were successfully used for the therapy of certain diseases in human patients (Dyckerhoff and Gaus, 1963).

We decided to employ enzymatic hydrolysates prepared according to Tushnov, the preparations had a stimulatory effect in low doses. Homologous and heterologous preparations from the hearts of rat, rabbit and dog were prepared. These hydrolysates were injected to the rats (6 μg per 300-400 gm body weight) and to the rabbits (60 μg per 3-4 kg body weight) immediately after the operation, followed by six single injections every other day. In addition to these preparations, high polymer RNA was injected, protein extracts from the cardial or skeletal muscle, thyreoidine, vitamin B_{12}, as well as pyrogenal, trypsin, leukocyte serum, dibazol and other drugs. Methods of preparation and administration, doses and other details are presented in our monograph (Polezhaev and coworkers, 1965).

Old, white, male rats, weighing 300 to 400 gm, and male rabbits (Chinchilla) weighing 3 to 4 kg, were used in all basic experiments. We also used mongrel dogs aging between two and five years. The animals were operated using the apparatus type DP-1 for artificial respiration under strictly aseptic conditions. Hearts were studied by various histological, histochemical, physiological and biochemical techniques at different times up to one year after the operation. All the details of this study were presented in our monograph (Polezhaev and co-workers, 1965).

The development of methods for the controlled damage of the heart muscle took two to three years. We made various attempts such as ligating of the left branch of the descending coronary arterium, burning, local application of the high vacuum or electrodiathermocoagulation. This latter procedure was most successful. It permitted the obtainment of small lesions having the shape of the conus 4 to 5 mm in diameter and with the same height, its top

was directed towards the endocardium. This lesion could be made at strictly definite places in our case near the top of the left ventricle.

We began these experiments in 1956. The first results were unexpected and demanded further improvements in the course of our studies.

STIMULATION OF HEART MUSCLE REGENERATION

In all experiments, experimental damage of the myocardium in rats, rabbits and kittens invariably leads to inflammation and tissue necrosis, followed by their lysis and substitution with granulation and then with dense scar tissue, no matter, whether the damage was made by burning with glowing platinum wire of electrothermocauter, experimental infarcture, by the local application of the vacuum or by the electrodiathermocoagulation (Fig. VI-3). Mus-

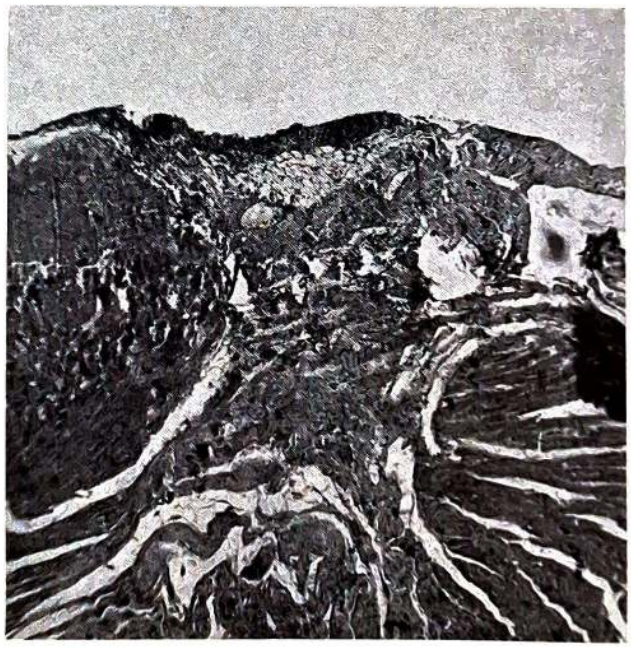

Figure VI-3. Focus of injury, 120 days after coagulation of rat miocardium. Connective-tissue scar, x 35.

cle fibers of the heart did not regenerate. The destruction of muscle stumps was only very weak, they did not dedifferentiate and were covered with closely fitting connective tissue (Fig. VI-4). The amitotic division of their nuclei occurred relatively frequently, while the mitoses were extremely rare. At the same time separate myoblasts and their chains were observed in the center of diathermoco-

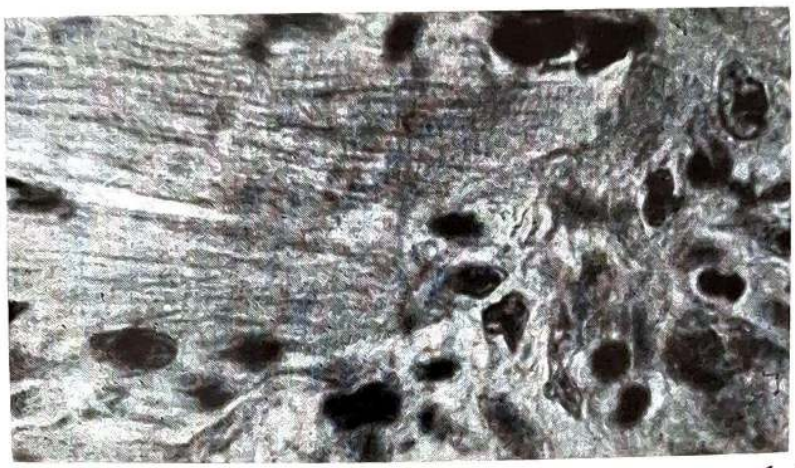

Figure VI-4. Muscle stumps in the 10th day after coagulation of rat miocardium. No dedifferentiation. Ocular 8, objective 100.

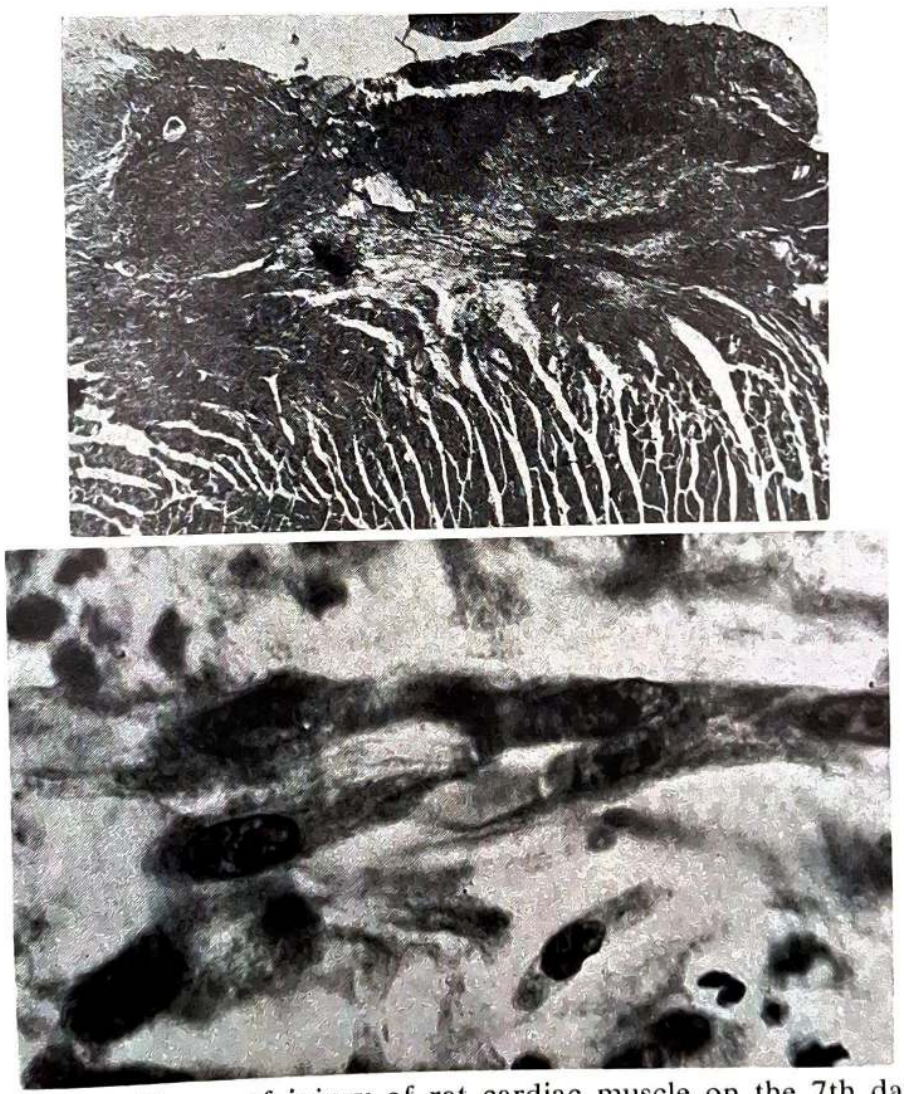

Figure VI-5. Focus of injury of rat cardiac muscle on the 7th day after diathermocoagulation. Top, General view of focus of injury. Microsummar, 20 mm, F:4.5. Bottom, Chains of myoblasts in the center of focus of injury. Ocular 10, objective 90.

agulation damaged region on the near of rapidly lysing tissues undergoing the necrosis. They were not connected with muscle stumps (Fig. VI-5). These cells possessed elongated clear nuclei, large nucleoli and long processes of basophilic cytoplasm. They frequently showed mitotic divisions (Fig. VI-6). They readily con-

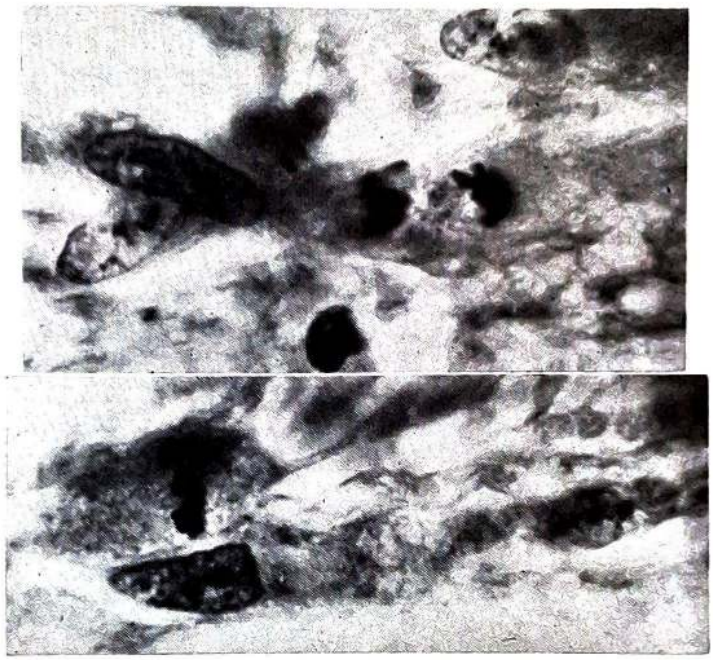

Figure VI-6. Proliferation of myoblasts in the focus of injury of rat cardiac muscle. Seven days after operation. Top and bottom, Mitoses in myoblasts. Ocular 10, objective 90.

nected with each other forming chains and syncytium. Many cells of polyblast type showing different stages of transition to myoblasts as judged from their structure and staining, were found between them. The origin of these remains unknown. It may be that they have myogenic origin splitting from muscle fiber stumps and migrating into the damaged region. On the other hand we cannot exclude at present that they are formed from other cells possibly polyblasts, which are transformed under the specific influence of muscle fiber degradation products, abundant in the damaged region. English authors (Batson, Woodrow and Sloper, 1966) studied skeletal muscle regeneration in white mice by autoradiography using thymidine-H^3. They concluded that myoblasts can originate from the white blood cells, but this required further investigation.

Later, twenty days after the operation, when the lysis of necrotized tissues is completed, myoblasts in the scar region, distant

from the muscle stumps, become organized into islands or bundles of atypical muscle fibers (Fig. VI-7A). These fibers are different from the typical heart muscle fibers. They have somewhat elongated nuclei, clear fine-grain sarcoplasm and form the syncytium. They have no myofibrils, have no cross-striation (or it is very weak) and have no intercalated disk (Fig. VI-7B).

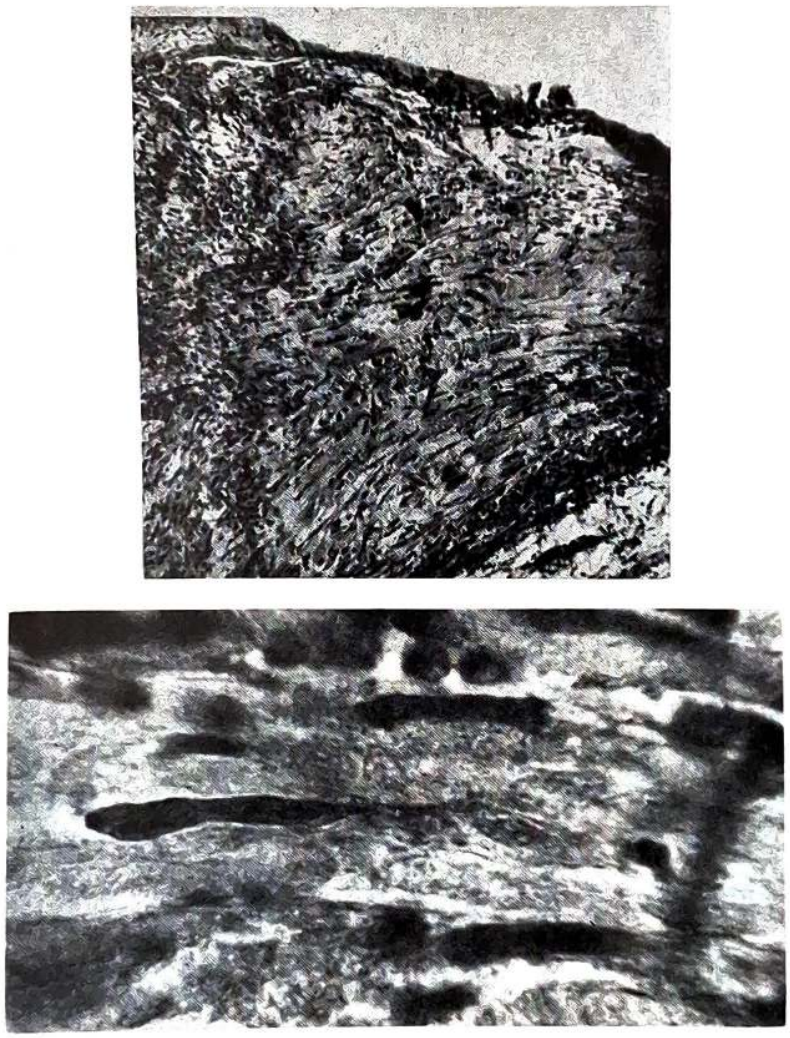

Figure VI-7. *De novo* formation of nondifferentiated muscle fibers in the focus of injury of rat myocardium on the 20th day after diathermocoagulation. Top, x 35. Bottom, Ocular 10, objective 8.

Compressed with dense maturating scar, these islands of new muscle tissue resolve and disappear 1 to 1.5 months after the operation.

A different picture is observed when heart muscle hydrolysate is injected into the animals with heart muscle damaged by diathermocoagulation. Under these conditions the lysis of necrotized tis-

sues accelerates about 2.5 times, stumps of muscle fibers undergo dedifferentiation, cross-striation and myofibrils disappear. Muscle buds or myoblasts are formed in stumps and abundant myoblasts are present in granulations (Fig. VI-8,9). These myoblasts grow and undergo conversion into the mass of new muscle fibers, which in some places completely fill the damaged region (Fig. VI-10A). However, as revealed by histological criterion they show no differ-

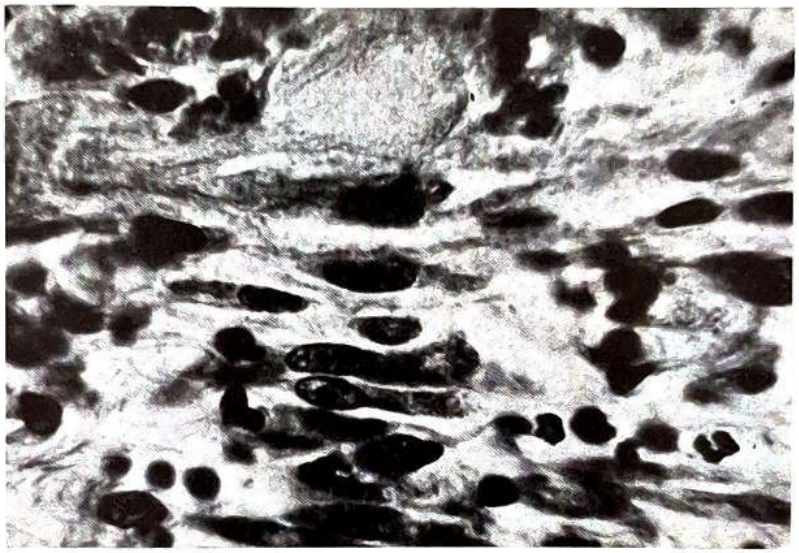

Figure VI-8. Dedifferentiation of muscle fiber stumps, formation of muscle bud on a stump and numerous myoblasts in granulation tissue. An experiment with hydrolysate of myocardium. Five days after operation. Ocular 8, objective 100.

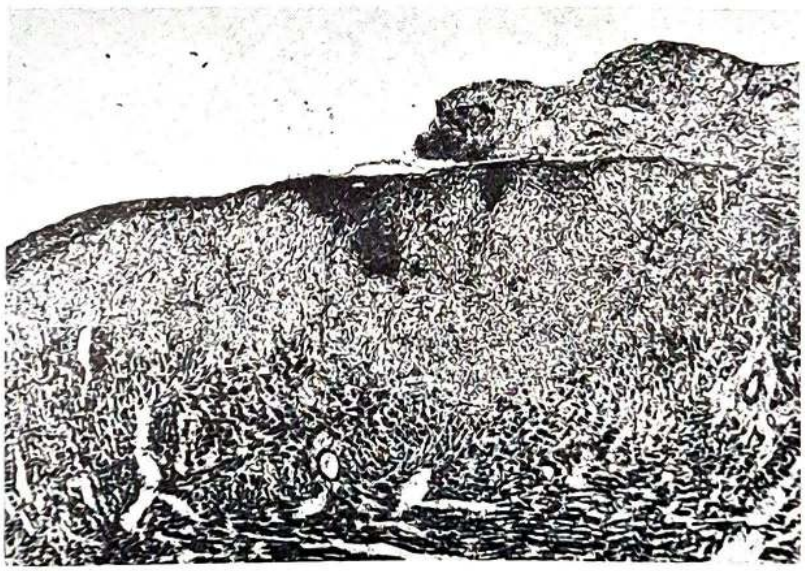

Figure VI-9. A focus of injury on the 5th day after diathermocoagulation. An experiment with hydrolysate of myocardium. Complete lysis of necrotized muscles. Granulation in a focus of injury. x 35.

entiation as the controls (Fig. VI-10B). These masses are separated from the muscle stumps by a dense scar of fibrous tissue. During the maturation the scar compresses this tissue and two months after the operation it undergoes resolution and is substituted by the scar. Testing of different preparations (heart muscle hydrolysate, protein-containing extracts of the heart or skeletal muscle, vitamin B_{12}, thyreoidine etc.) has shown that they can also stimulate the formation of new undifferentiated muscle tissue in the damaged area and stump dedifferentiation in the border zone, but their activity and mode of their action is somewhat different. Heart

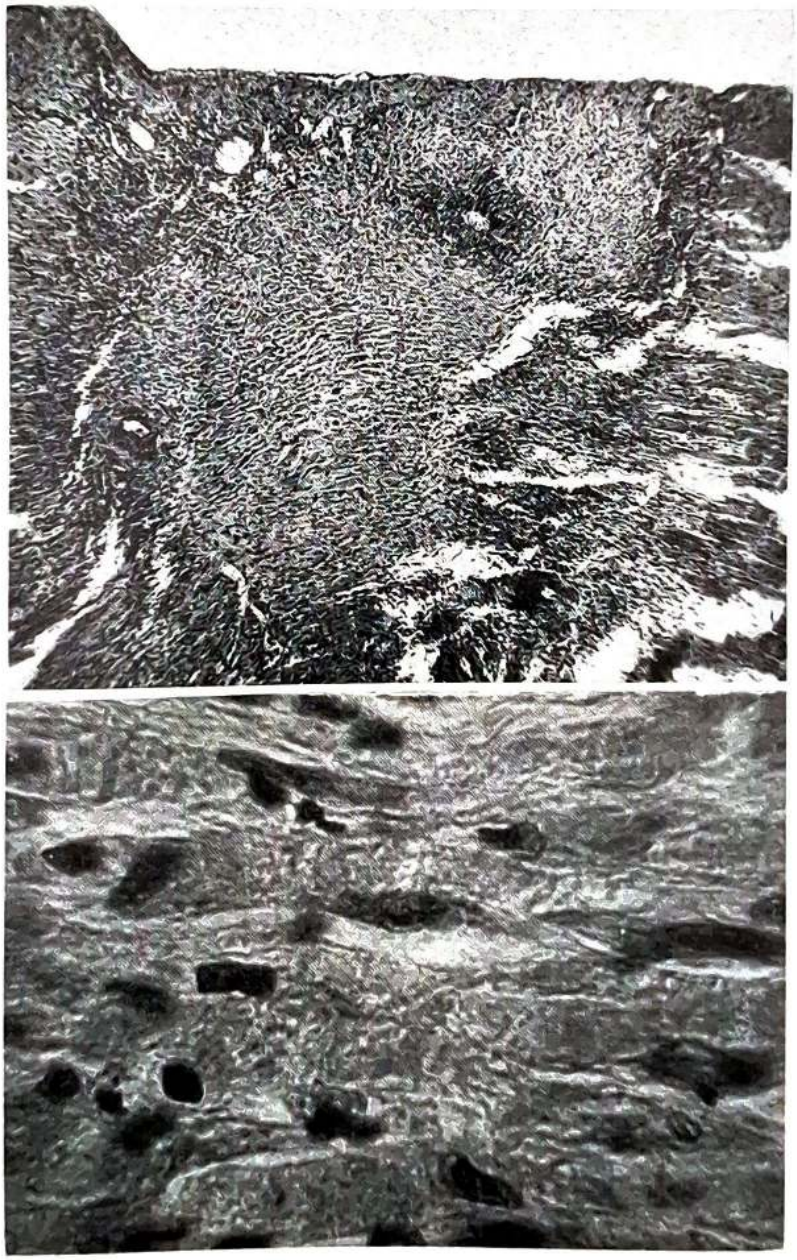

Figure VI-10. *De novo* formation of muscle stumps in a focus of injury in the rat myocardium thirteen days after diathermocoagulation. Top, General view of a focus of injury; x 40. Bottom, Undifferentiated de novo formed muscle fibers. Ocular 8, objective 100.

muscle hydrolysate has the strongest action. However, under all conditions, new heart muscle undergoes resolution under the effect of the developing dense scar.

Application of RNA and dibazol leads to some decrease of undifferentiated muscle fiber formation in the damaged region. But even in this case muscle buds are formed at the stumps as well as myoblasts, and muscle fiber processes which differentiate further (Fig. VI-11,12,13). This growth, however is very restricted and

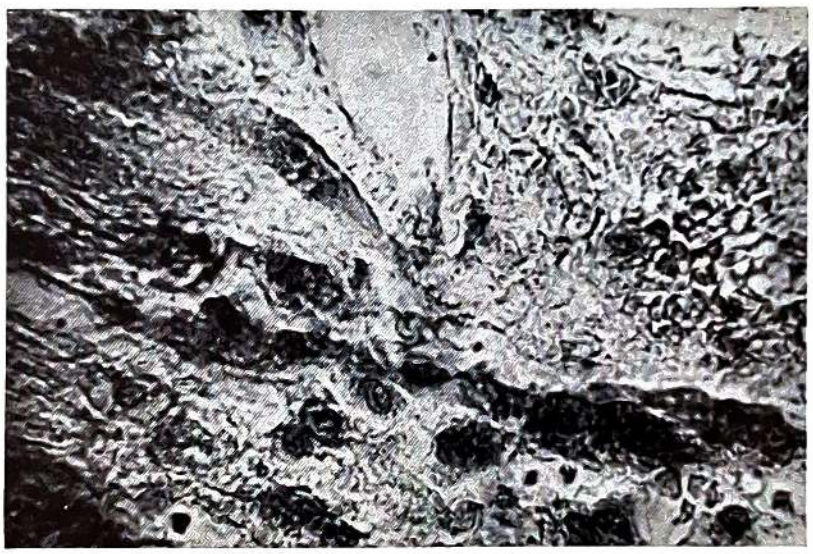

Figure VI-11. Formation of myoblasts and muscle outgrowths on muscle stumps. An experiment with RNA. Three days after operation. Ocular 10, objective 60.

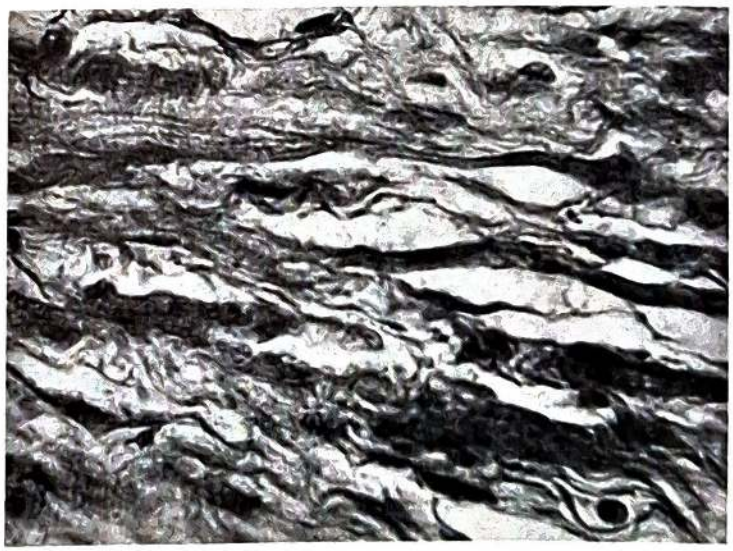

Figure VI-12. Formation of outgrowths on stumps of dedifferentiated rat heart muscles. An experiment with RNA. Thirteen days after operation. Ocular 10, objective 60.

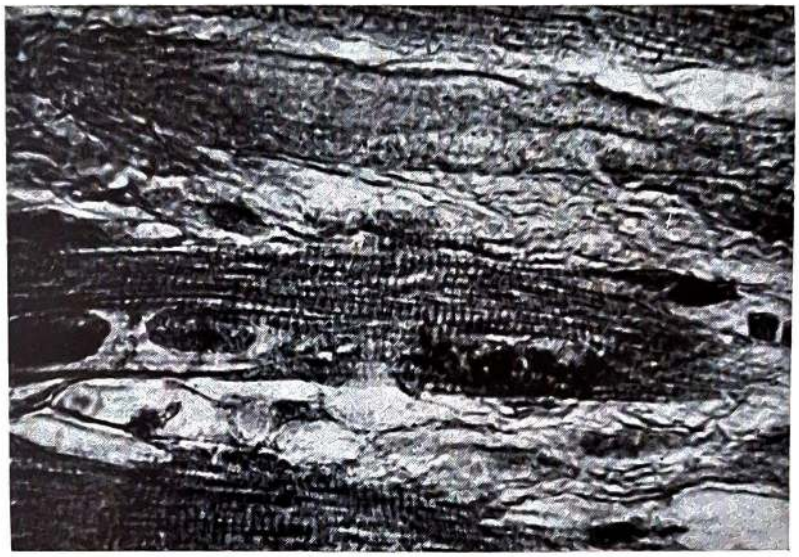

Figure VI-13. Regeneration of nondifferentiated muscle fibers on muscle stumps. Amitotic nuclei division in a differentiated muscle fiber. An experiment wth RNA. Twenty-one days after diathermocoagulation of myocardium. Ocular 10, objective 60.

muscle fiber regeneration from the stumps can be observed only at the very border of the damaged region.

EXPERIMENTS ON THE INHIBITION OF SCARRING

Our task was to influence the development of the connective tissue in the heart muscle damaged region in such a way as to inhibit the scarring and to soften the scar. This would diminish its pressure on the new muscle fibers and facilitate their growth through the scar, which would lead to their survival. Such a mode of action is expected from the hyaluronidase but when injected into the bloodstream it undergoes rapid inactivation even before it comes into contact with the damaged heart muscle. Therefore we used other drugs in our experiments. These were the following: pyrogenal, preparation from the spleen, trypsin and dibazol. Pyrogenal is a lipopolysaccharide of bacterial origin (Budnizkaya, 1961) an analog by Pyromen, which was successfully used by American investigators for the inhibition of glial scar formation after the cutting of the spinal cord (Windle, 1955). Similar action was noted for trypsin (Turbes and Freeman, 1953; Freeman, *et al.*, 1960). Preparation from the spleen shows favorable results in cases of blindness, deafness and sterility, caused by inflammatory processes, affecting visual and auditory nerves, or leading to oviduct obliteration (Rumyantsev, 1951, 1953).

The ability of dibazol to inhibit scar formation was established in our experiments (Polezhaev, *et al.*, 1960).

When we injected these preparations into the rats or rabbits with damaged heart muscle we were able to retard the scarring and to alter the structure of the scar. Administration of pyrogenal leads to a drastic decrease of tissue infiltration by the cellular elements of the white blood. Only a few granulocytes and lymphocytes are observed in the damaged region; a much smaller number of polyblasts, hystiocytes and fibroblasts are found at later stages. Probably pyrogenal leads to the decrease of vessel wall permeability. The formation of collagen fibers takes place very early after the damage, they are thick but packed in a rather loose manner, so that there are many interstitial spaces which can be occupied by the muscle fibers (Fig. VI-14). The scar in this case is not so

Figure VI-14. Collagen fibers and gaps between them in a focus of injury of rat cardiac muscle. An experiment with pyrogenal. Ninety days after operation. Ocular 10, objective 60.

dense and does not exhibit such strong pressure upon the muscle stumps as in controls, when no drugs were given after the diathermocoagulation. The effect of preparation from the spleen is similar, but the damaged region is more heavily invaded by granulocytes and lymphocytes, while collagen fibers are interwoven in a more dense manner than in experiments with pyrogenal. After the trypsin administration the scar is very dense at the late stages. In experiments with pyrogenal administration very thin basophilic processes sometimes containing amitotically dividing nuclei were grow-

ing from the muscle stumps in certain areas of the damaged region mainly at the periphery and near the large vessels (Fig. VI-15). These processes penetrated into the clear spaces between the scar collagen fibers, differentiated and remained for long periods of time, sometimes for a few months. Bridge formation was sometimes observed. But the mass of the regenerated muscles is small. The administration of scar formation inhibitors results in considerable decrease of new muscles in the damaged region.

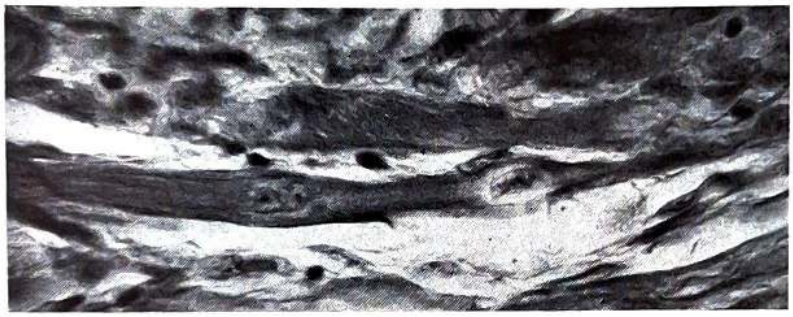

Figure VI-15. Muscle outgrowths regenerating on muscle fiber stumps with amitotically dividing nuclei. An experiment wth pyrogenal. Ten days after operation. Ocular 10, objective 60.

COMPLEX USE OF HEART MUSCLE REGENERATION STIMULANTS AND SCAR INHIBITORS

In order to stimulate the growth of new muscle in the damaged region and to achieve their prolonged survival we used different combinations of regeneration stimulants and inhibitors of scar formation and tested them in experiments with rats and rabbits. The following combinations were tested: heart muscle hydrolysate (HMH) plus pyrogenal; HMH plus a preparation from the spleen; HMH plus trypsin; vitamin B_{12} plus pyrogenal; RNA plus pyrogenal; RNA plus a preparation from the spleen; HMH plus dibazol plus vitamin B_{12} and others. It was found that different combinations had different activity, in addition, the effects on the rat and rabbit heart might be also different. In some cases the effects of individual components were neutralized during the joint application, while in other cases the action of combination was stronger than that of individual agents. Such successful combinations lead to more pronounced heart muscle fiber regeneration, which was more stable than with stimulants or inhibitors only.

In experiments conducted on the rats administration of HMH plus pyrogenal or HMH plus a preparation from the spleen re-

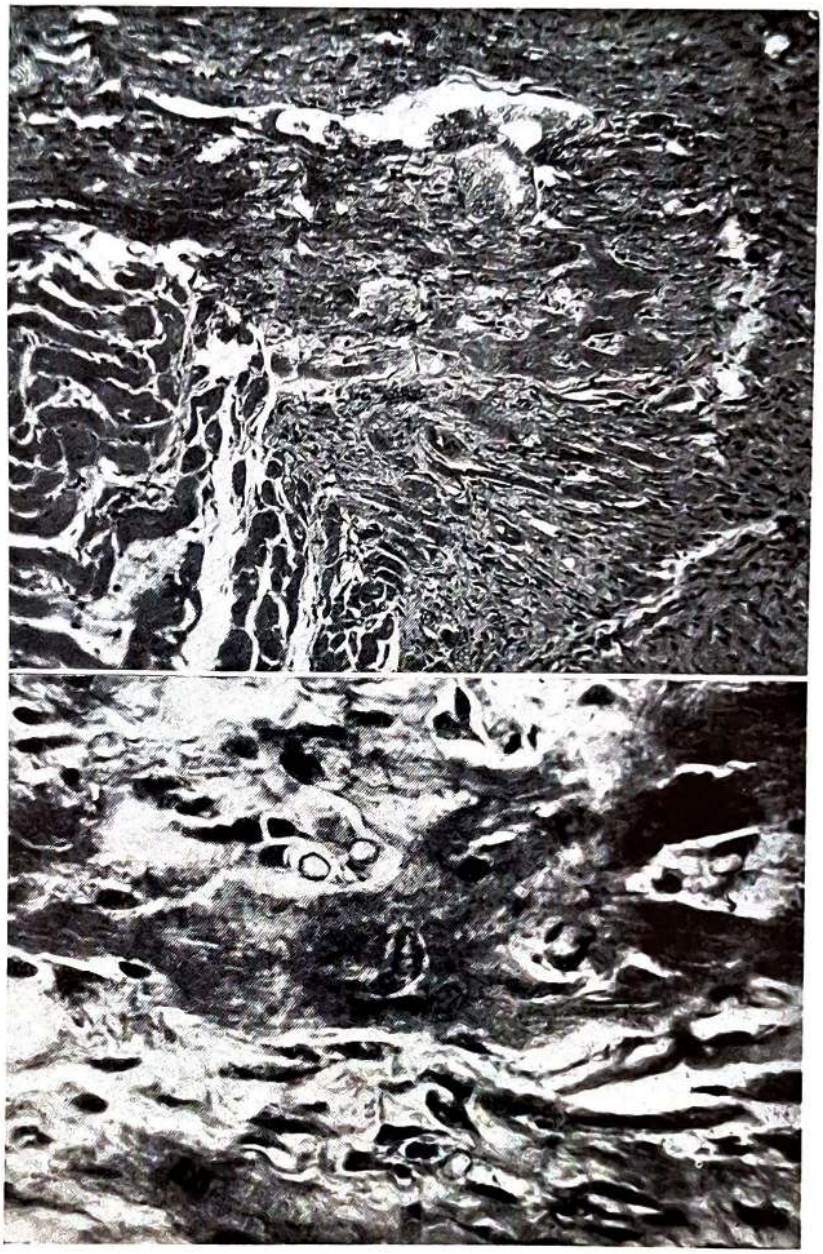

Figure VI-16. *De novo* formation of muscle fibers in a focus of injury of rat heart. An experiment with hydrolysate of myocardium + vitamin B_{12} + dibazol. Twenty-one days after operation. Top, A general view of the focus of injury; left—old muscle fibers; middle—new muscle fibers; ocular 7, objective 20. Bottom, New muscle fibers ocular 10, objective 60.

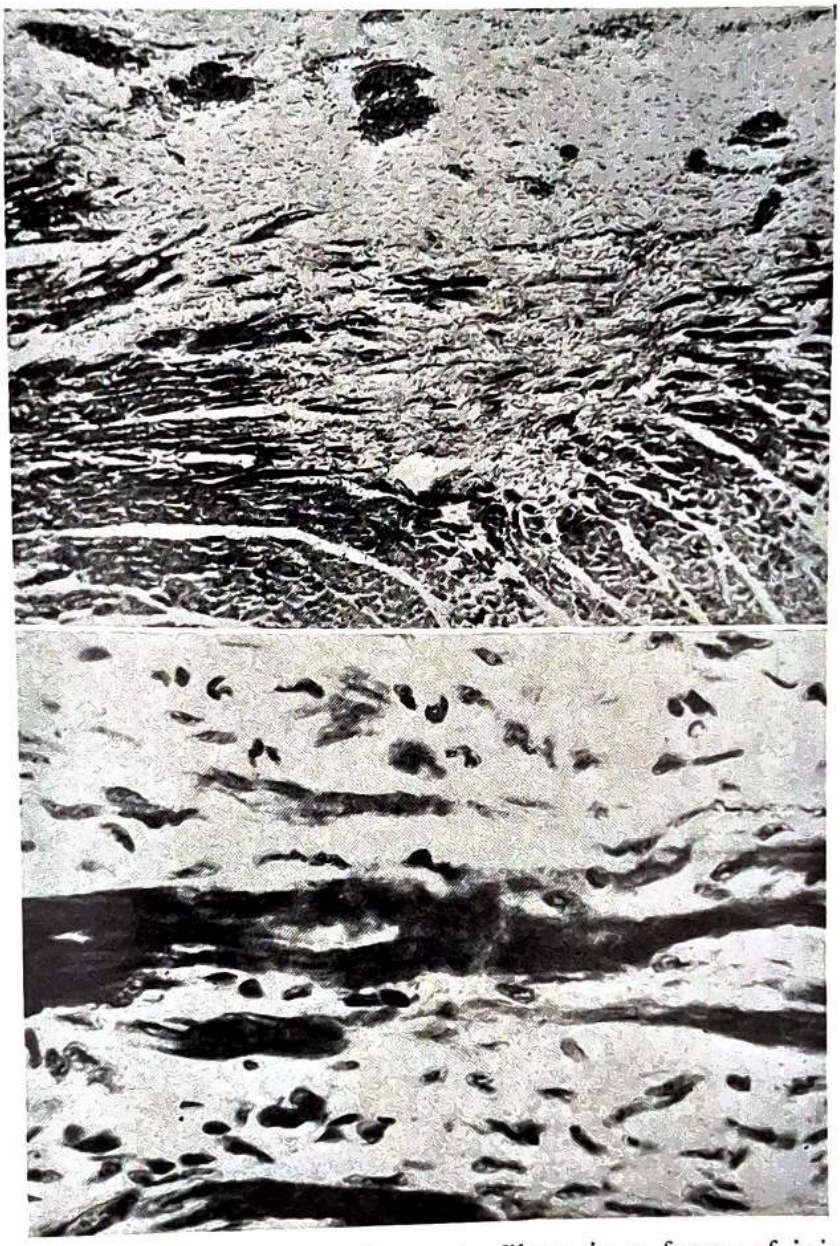

Figure VI-17. Regeneration of muscle fibers in a focus of injury of rat cardiac muscle. An experiment with hydrolysate of myocardium + pyrogenal, 158 days after operation. Top, A general view of a focus of injury; muscle bridgework; ocular 7, objective 8. Bottom, Separate regenerated muscle fibers at the focal margin. Ocular 10, objective 60.

sulted in rapid filling of the damaged area (ten to twenty days) with new undifferentiated muscle fibers. However, dense scar formed at the edge of the damaged zone (Fig. VI-16). Later, for example, thirty days after the operation, muscle regeneration in the border zone took place, and at even later times associations or bridges of regenerated muscle fibers were formed (Fig. VI-17,18). However, after a certain delay these new muscle fibers located at the center of the damaged region resolved and only those muscle fibers, which regenerated at the periphery of the damaged area remained.

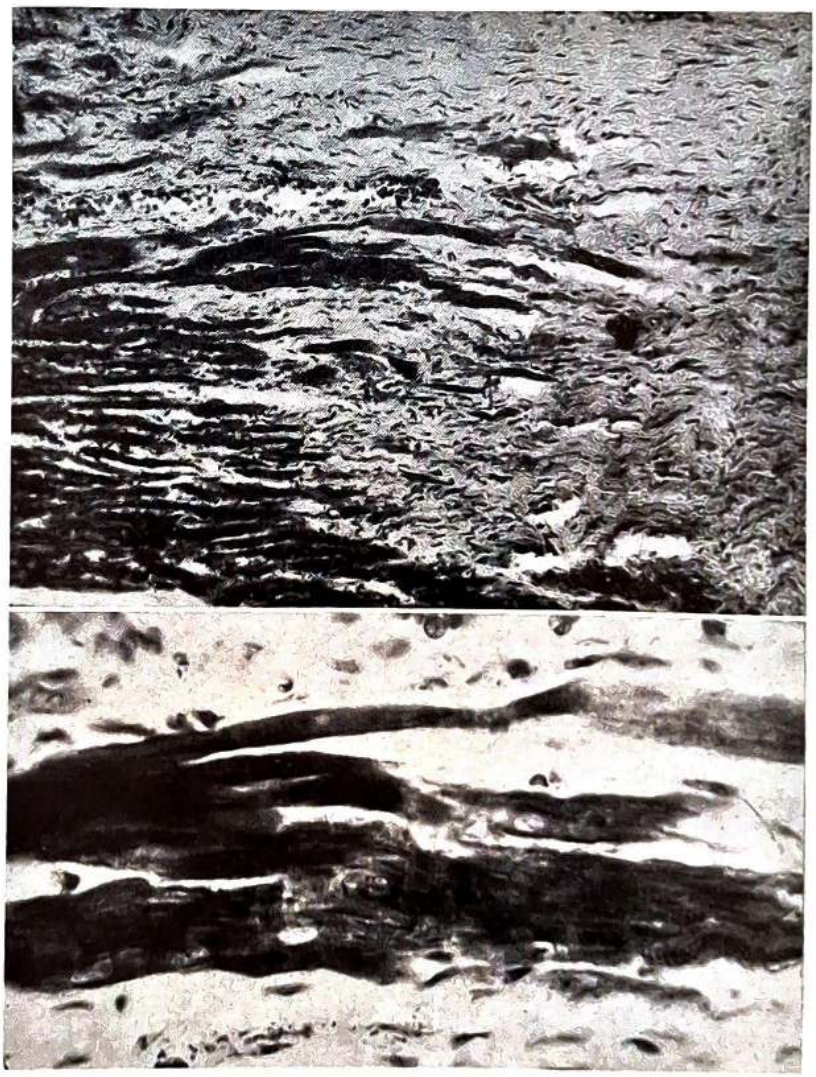

Figure VI-18. Regeneration of muscle fibers in a focus of injury of rat cardiac muscle. An experiment with hydrolysate of myocardium + pyrogenal, 158 days after operation. Top, a general view of a focus of injury, ocular 5, objective 10x. Bottom, Regenerated muscle fibers. Ocular 7, objective 60x.

HEART MUSCLE REGENERATION BY INDUCTION

Regeneration of muscle fibers from the stumps was only very limited in our experiments. It occurred by the growth of myogenic elements from the muscle stumps. The origin and mechanism of formation of nondifferentiated muscle fibers in the center of the damaged zone, independently of the stumps, was obscure. These fibers might originate from the myogenic elements, which splitted from the muscle stumps and migrated to the center of the damaged zone. This is confirmed by the existence of "myogenic granulations" (Oppel, 1901; Anitschkow, 1913, 1913a). On the other hand we cannot exclude that myoblasts are formed from some other cells (polyblasts possibly) under the influence of the specific action of heart muscle degradation products in the damaged region. This possibility follows from the existence of certain transition forms of cells intermediate between polyblasts and myoblasts in the center of the damaged region. The atypical structure, absence of differentiation and final disappearance of these fibers from the lesion also lend some support to this possibility. Their formation can be explained on the basis of observations of Levander (1964), who obtained muscle induction in the subcutaneous connective tissue or omentum of the rabbit, implanting there pieces of necrotized skeletal muscles, treated for twenty-four to forty-eight hours in 1% water solution of trypan blue.

In our experiments we followed the procedure of Levander: pieces of rabbit femoral muscle were treated in 1% water solution of trypan blue and then implanted into the muscle of rabbit heart left ventricle. The implant was completely necrotic and devoid of nuclei. In granulations formed in its immediate vicinity it induced myoblasts and chains of myoblasts, which then gave rise to thick basophilic muscle fibers, forming the syncythium and containing abundant nuclei (Fig. VI-19). Myoblasts were formed from the polyblast-like cells. The induced muscle fibers developed in complete independence from the muscle stumps of the border zone and were undifferentiated. After twenty-five to thirty days they showed the symptoms of atrophy, underwent degradation and were finally substituted by scar tissue.

When steel or gelatin needles are implanted into the heart muscle, a zone of degradation is formed around, followed by the scar

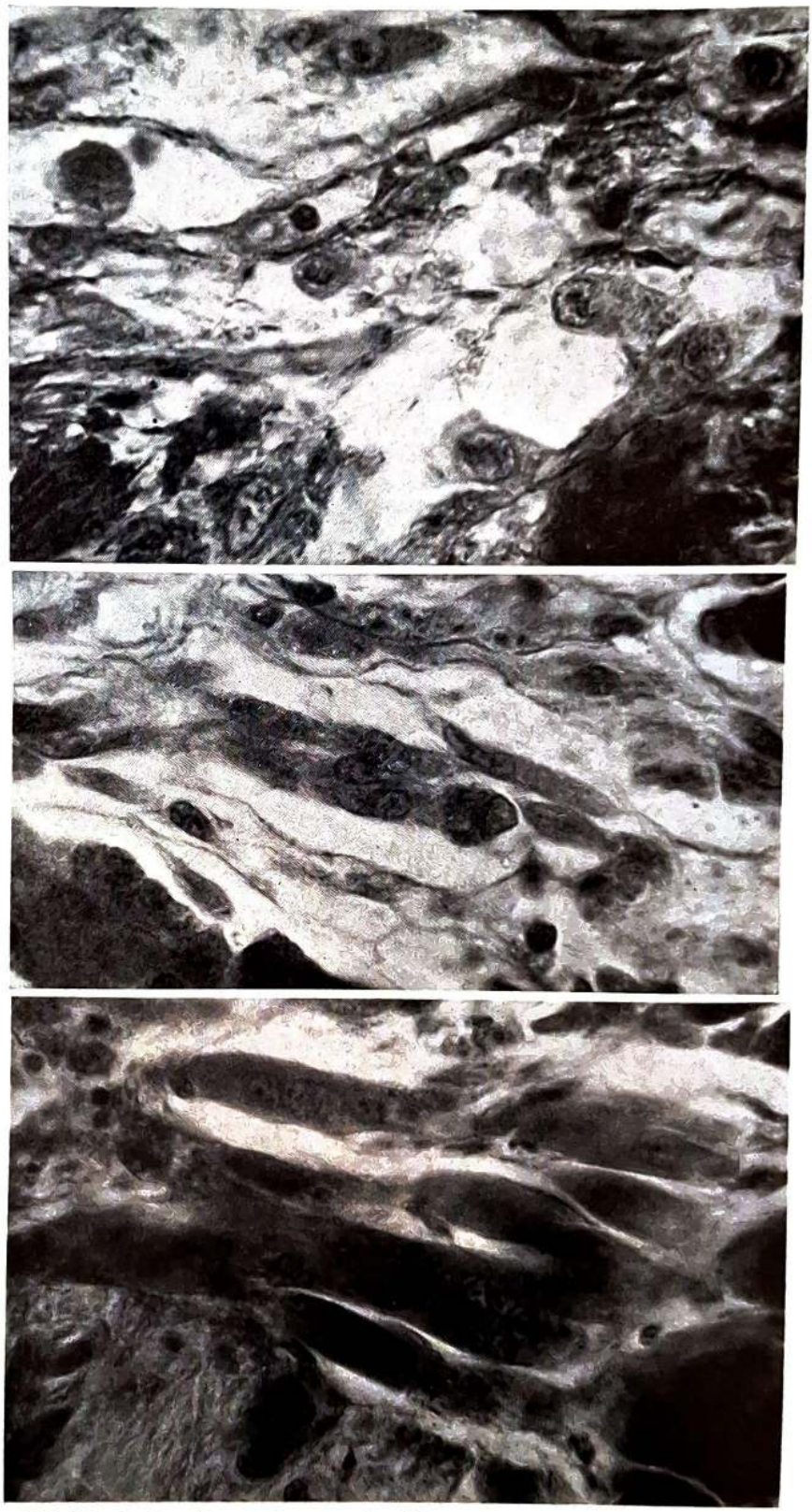

Figure VI-19. Induction of muscle fibers in the rabbit heart by grafting a piece of skeletal muscle treated according to Levander. Ocular 10, objective 60. Top, Polyblasts with basophilic outgrowths, three days after operation; Middle, Chains of myoblasts, five days after operation; Bottom, Muscle syncytium, basophylic sarcoplasm, nuclei clustered, twenty-four days after operation.

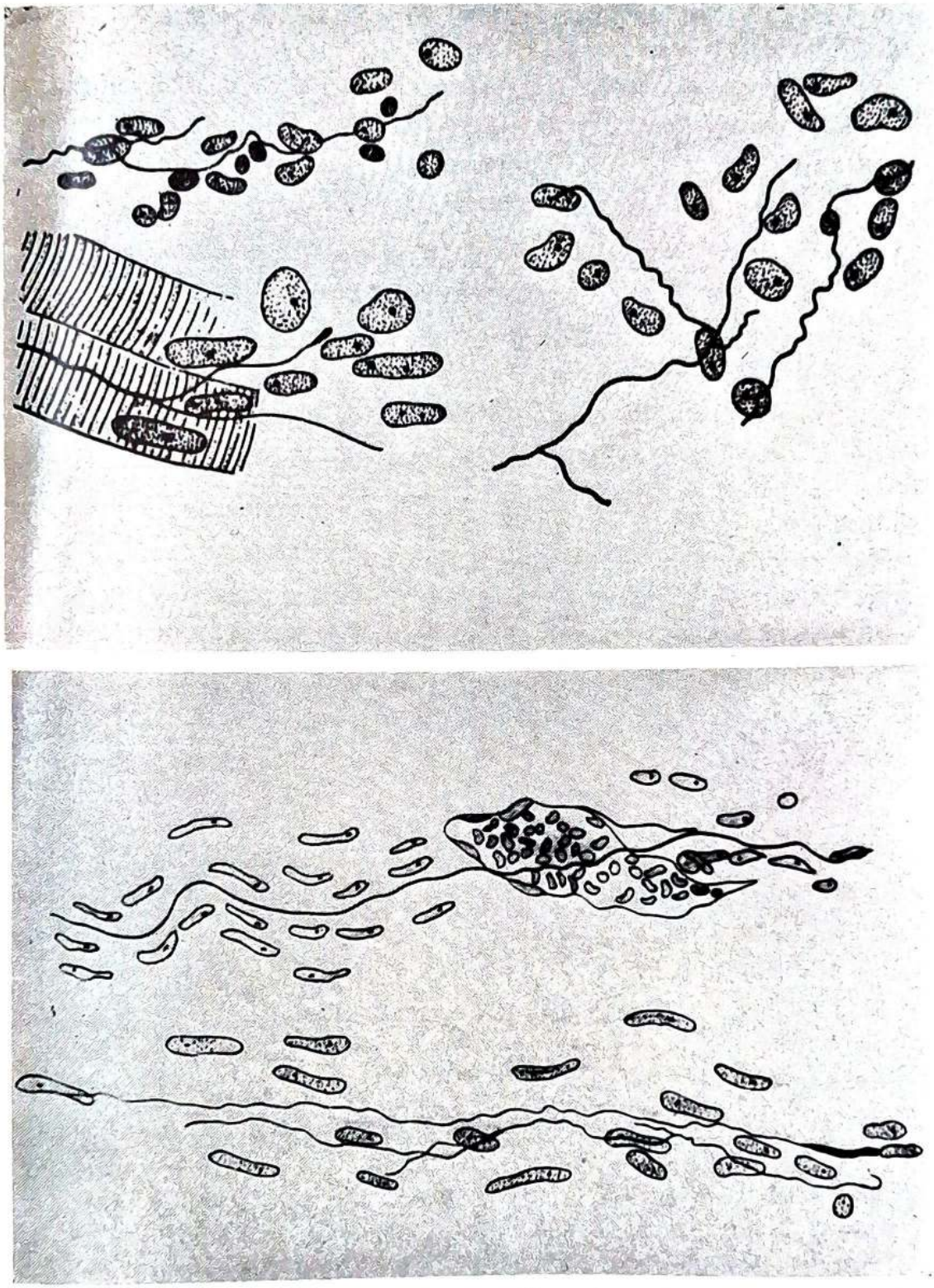

Figure VI-20. Regeneration of nerve fibers in a focus of injury in the rat heart. Top, seven days after operation; Bottom, sixty days after operation (Gusareva, 1964).

formation (Oppel, 1901; Anitschkow, 1913, 1913a). Muscle induction is not observed.

Our results demonstrate the possibility of heart muscle induction from the cellular elements of myocardial granulations, but they do not exclude that *myogenic* elements may participate in the regeneration of heart muscle fibers.

RESULTS OBTAINED IN HISTOCHEMICAL, NEUROHISTOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL INVESTIGATION

It has been shown, using Brachet reaction, that RNA is distributed predominantly in young structural elements of the heart, showing high ability to regeneration, that is in myoblasts, new muscle fibers, dedifferentiating muscle stumps, their processes, threads and bridges. In myoblasts and undifferentiated muscle fibers RNA is located in cytoplasm and in nucleoli. In heart muscle fibers the intensity of reaction for RNA is higher than in the fibrous tissue.

Results of Feulgen reaction show that DNA is localized in the nuclei of muscle fibers, cells of fibrous tissue and leukocytes. In addition till the forty-fifth day after the operation it is revealed in the nuclear debris and intercellular substance of the damaged region. Similar results were reported by Banting (1958) as well as Dumont (1959) and Ageev (1961).

Glycogen in the normal heart muscle fibers is distributed in a diffuse manner as granules; it is also found in myofibrils and Q discs. It also shows a diffuse distribution pattern in myoblasts and regenerated muscle fibers both undifferentiated and differentiated. In the latter case it is found at only one surface of the fibril, as in the normal state.

Neurohistological investigation conducted by Gusareva (1964, 1965) has shown that within ten to thirteen days after the operation all the damaged lesion becomes penetrated with a dense network of nerve fibers and nerve endings (Fig. VI-20). When myocardium regeneration is stimulated, the processes regenerating from the muscle stumps become innervated as well as new undifferentiated muscle fibers at the center of the damaged lesion (Fig. VI-21). This permits the following conclusions a) the absence of regeneration from the muscle stumps is not accompanied with the absence of their innervation; b) the absence of differentiation of new muscle

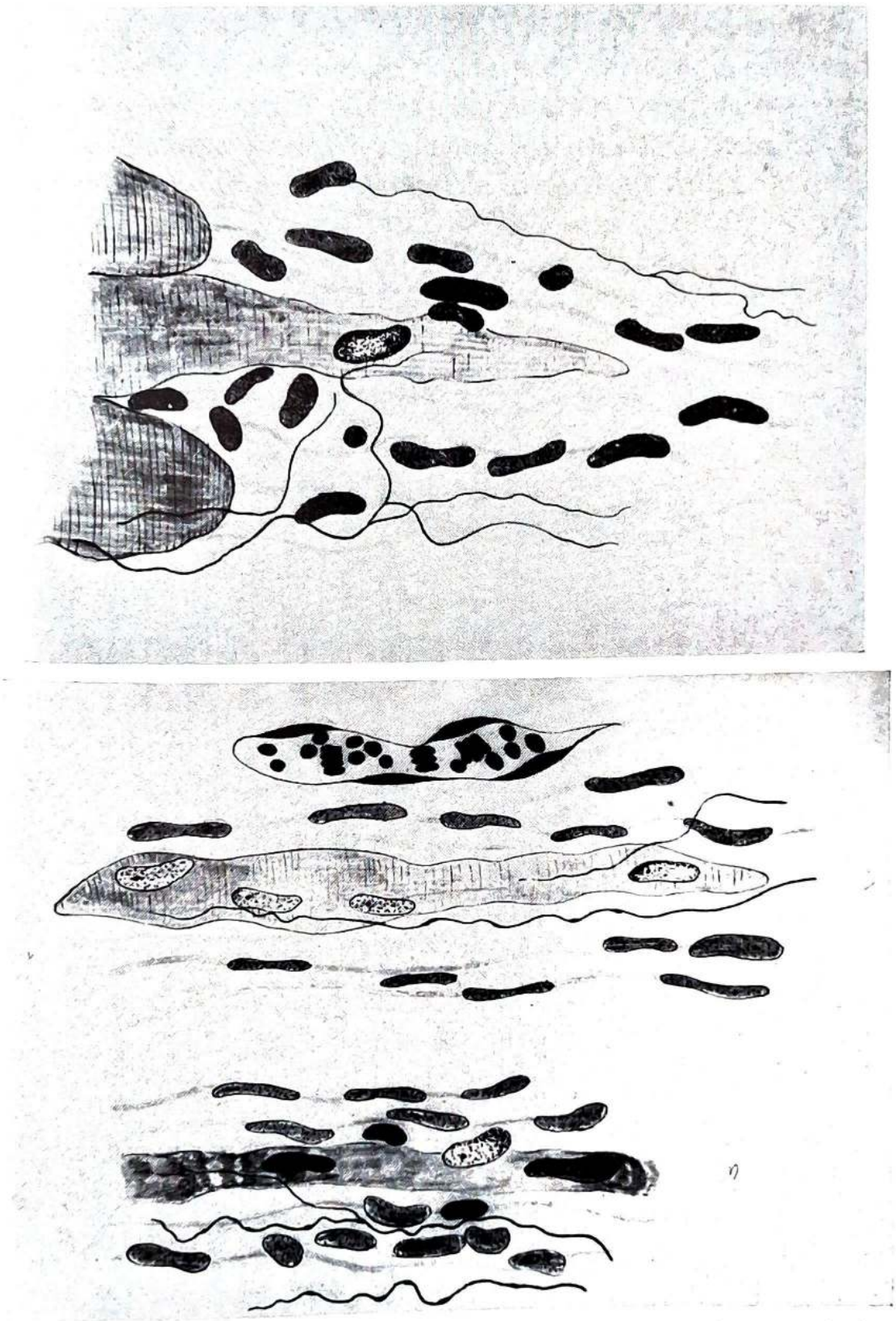

Figure VI-21. Regeneration of nerve fibers in rat cardiac muscle by stimulating its regeneration. An experiment with hydrolysate of myocardium + RNA. Top, Innervation of regenerating muscle fiber in a marginal zone, seventeen days after operation. Bottom, Innervation of undifferentiated *de novo* formed muscle fibers in a focus of injury; sixty days after operation (Gusareva, 1965).

fibers at the center of the damaged region depends not on the absence of their innervation but on some other factor.

After the heart muscle damage the electrocardiogram shows three consecutive types of the pattern—acute, subacute and scarring (Fig. VI-22). During the degradation of regenerated muscles the ECG returns from the norm to the scarring stage (Fig. VI-23). Stimulants of heart muscle regeneration and inhibitors of scarring have different effects on the shape of ECG pattern and dynamics of its changed as well as upon the per cent of returns to the normal state.

Biochemical studies have shown, that there is a certain change of protein and nucleic acid metabolism in the affected area after the myocardial damage and during the stimulation of its regeneration.

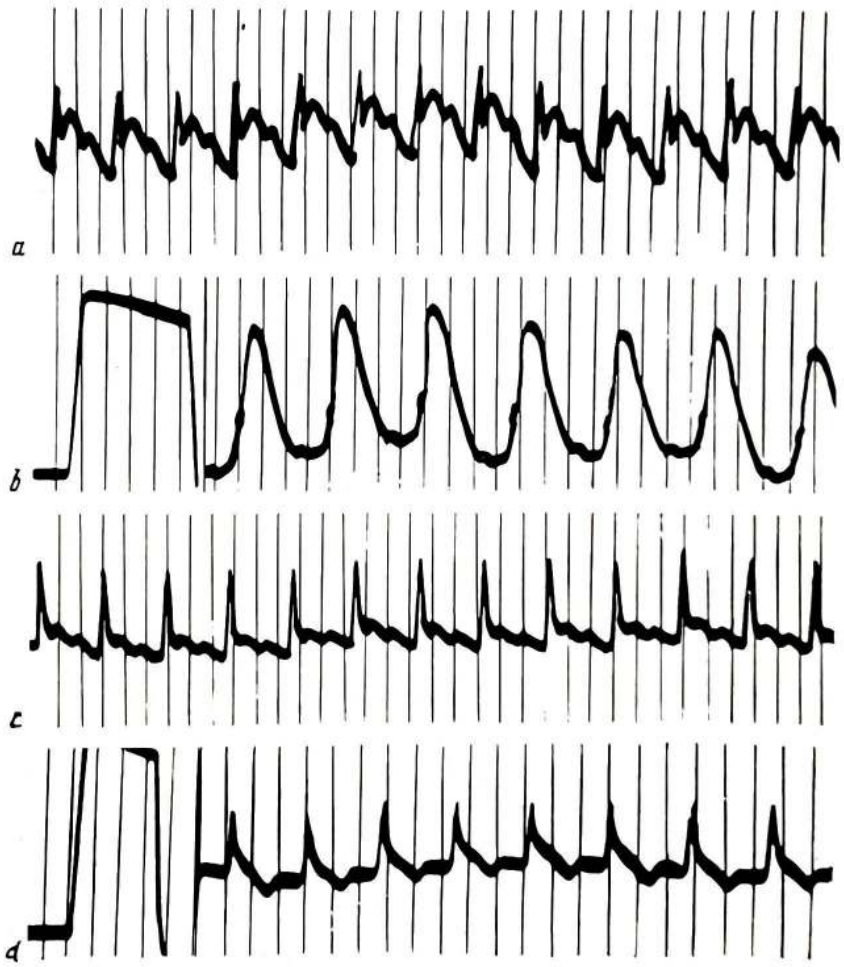

Figure VI-22. Electrocardiogram of rat in various times after injuring the myocardium. a. Normal. b. Acute phase. c. Subacute phase. d. Scar phase.

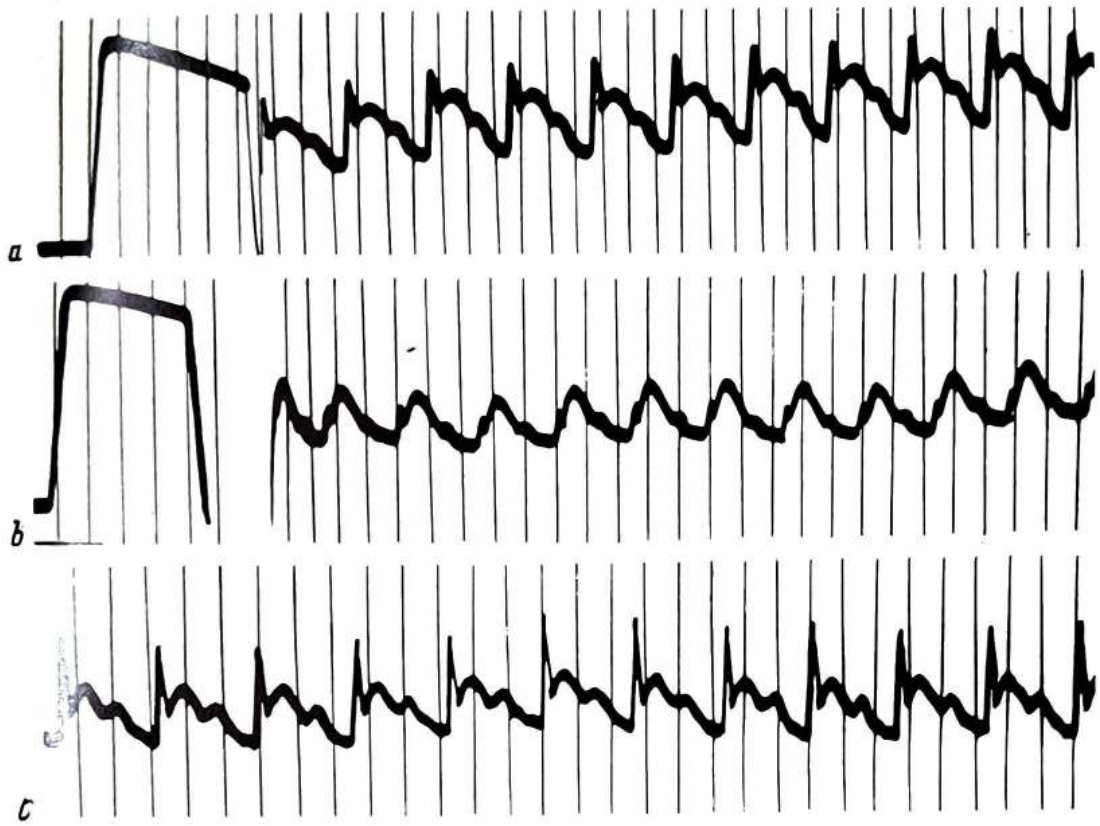

Figure VI-23. Electrocardiogram of rat after injuring the myocardium and stimulation of its regeneration. a. Chest lead in the control. b. Acute phase. c. Normalization on the 13th day after operation in the experiment with hydrolysate of myocardium.

There are only slight changes in the tissues distant from the damaged region. Heart muscle hydrolysate activates protein metabolism, predominantly protein synthesis. Vitamin B_{12} administration increases DNA and RNA content in the tissues of the damaged region, it also stimulates the incorporation of glycine (C^{14}) into proteins the fourteenth day after the operation. Pyrogenal administration stimulates protein synthesis in the damaged tissues at a later time, thirty days after the operation.

LITERATURE ABOUT HEART MUSCLE REGENERATION

Our first results about the possibility of heart muscle regeneration in adult mammals (Polezhaev *et al.*, 1958; Akhabadze, 1958; Zakharova, 1958; Mantieva, 1959) have been confirmed in a number of laboratories.

Sinitsyn (1959, 1960, 1961, 1966, 1968, 1969) removed large areas of heart muscle (up to 16 sq. cm) in dogs, ventricle cavities

were opened. If after the removal the edges of the wound were tightly sutured together and no regeneration was observed, the wound was healed with a scar. If the wound was covered with a

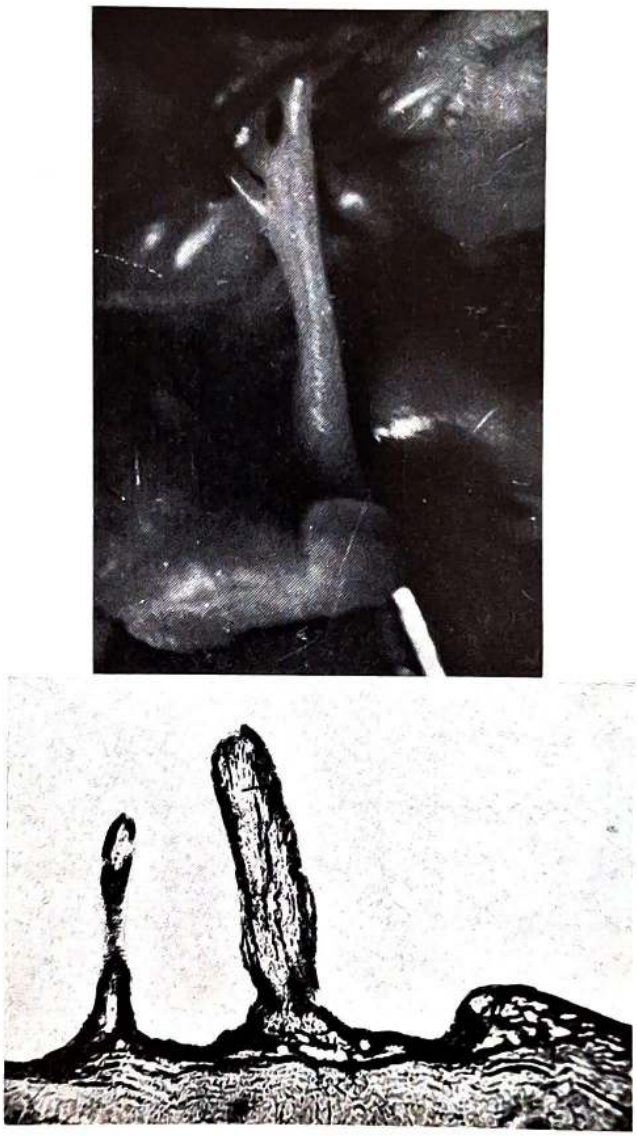

Figure VI-24. Regeneration of papillary cardiac muscle in adult dog after partial resecting the right ventriculum, Sinitsin, 1970). Left, A view of the heart from endocardium. Seventy days after operation. Below is horizontally-situated thick papillary muscle; it is pulled downwards by a silk loop. A large stalk grows upwards perpendicular to the muscle (in the center). Ramified into three branches, it intergrows into the resection zone of the right ventriculum. Right, A histological section of regenerating papillary cardiac muscle. One hundred days after operation. Van Gieson staining. Below is papillary muscle, from which upwards grow three vertical muscle stalks directed to the resection zone of the right ventriculum. During ninety days, the latter was subjected to the action of induced current (1.25 volts).

patch made of fascia, cloth, caprone or suède, then the wound was healed with a scar but on its inner side corresponding to endocardium and the wall thrombus, regeneration of well-differentiating heart muscle fibers were observed in all cases. Administration of vitamin B_{12} and dibazol stimulated heart muscle regeneration. It is interesting that muscle regeneration was independent of the stumps of the border zone. Good regeneration of papillary muscles was very remarkable (Fig. VI-24).

We repeated experiments of Sinitsyn with dogs and confirmed his results (Fig. VI-25, 26).

Kochetov (1959, 1961, 1966), imitating a landslide, subjected soft tissue of rabbit thigh to a strong mechanical pressure. As a result, multiple small lesions with the degradation of muscle fibers appeared in heart muscle of the animal. These lesions were of two types—granular and lump of a larger size. When the lesions were small and included small parts of one to three muscle fibers, these latter showed regeneration. When the lesions were larger, they healed with fibrous scar tissue.

Zaprjagaev (1969) obtained similar results in experiments with rabbits, which were placed under the conditions leading to ortho-

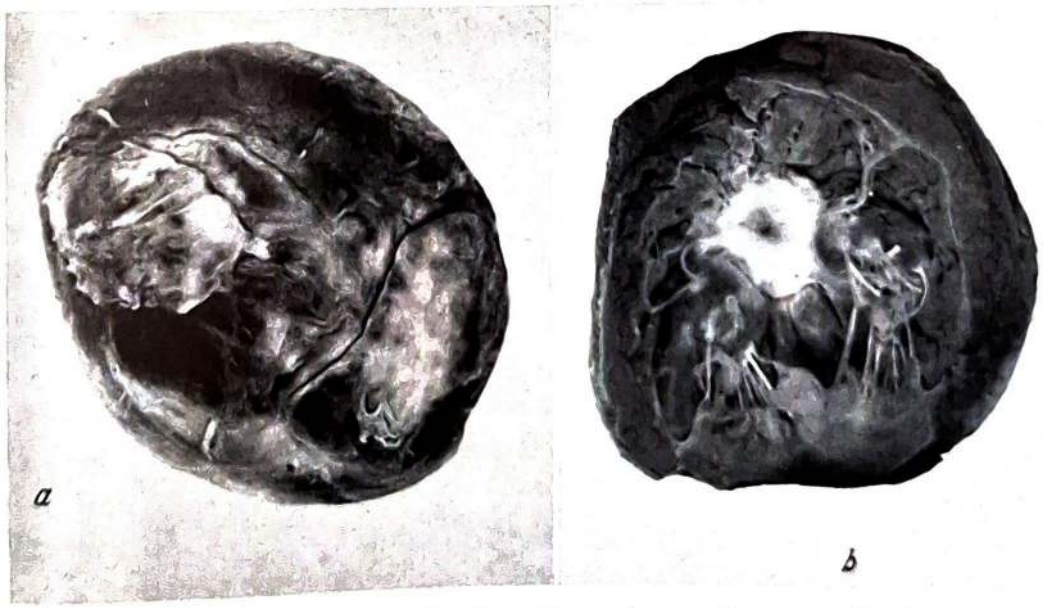

Figure VI-25. The heart of a dog. Resection and myocardioplasty according to N.P. Sinitsin, 315 days after operation. Left, External view; zone of resection seen on the upper left. Right, Internal view: in the middle, zone of resection where regeneration of muscle fibers occurred.

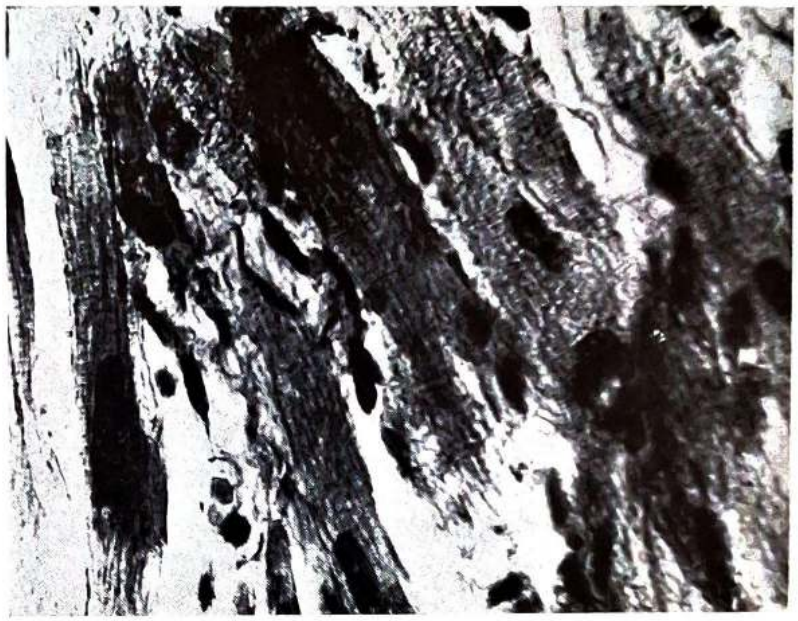

Figure VI-26. Packs of *de novo* formed muscle fibers in the scarf formed in the zone of resection and cardioplasty in dog, 315 days after operation. Ocular 10, objective 60.

static collapse—rabbits were tied to the support in the vertical position.

Andreev *et al.* (1968) and Saidrasulov (1963) made cut and stab wounds in hearts of rats and rabbits. These were healed with fibrous scar tissue. Administration of special therapeutic complex (vitamins B_1, B_6, B_{12}, ATP, methionine, RNA and DNA) which promoted protein synthesis, stimulated the regeneration of heart muscle fibers.

Glagoleva and Chechulin (1968) tied the left descendent branch of the coronary arterium in rabbits and followed the development of the infarcture using electron microscopy. It was found that myoblasts with myofibrils were formed in the region of the infarction. The administration of the therapeutic complex mentioned above stimulated the formation of myoblasts and muscle fibrils in the damaged region.

Naumets (1966) and Survillo and Naumets (1966) conducted a homotransplantation of lyophilized muscles into the dog myocardium. The resolution of these transplants was accompanied by the regeneration of muscle fibers in the recipient heart.

Nepomnjashchikh (1966) conducted an autotransplantation of minced pieces of skeletal and heart muscle into the dog myocardium. Myoblasts and muscle tubules were formed in the implant

region. Their further development was prevented by the formation of a dense scar.

Vitkus and Danelus (1966) obtained experimental infarctions in rabbits. Administration of protein hydrolysate prepared from the blood fibrin of cattle stimulated the lysis of necrotized tissues and regeneration of heart muscle in the necrotic zone. Some other preparations produced similar effect. Vitkus (1969) employing the cytophotometric method was able to show that in experiments with rabbit heart under these conditions, administration of stimulants increases DNA synthesis in muscle stump nuclei, leads to their polyploidization, which initiates their amitotic division and stimulates RNA synthesis in the stumps.

Rumjantsev and Mirakian (1968) observed considerable stimulation of DNA synthesis and mitotic divisions of muscle fiber nuclei in the auricle after the damage of myocardium in rats (infarcture, burn, smash).

Skuba (1965, 1968) damaged rat heart muscle by burning. He came to the conclusion that this resulted in the formation of myoblasts. Later he tried to differentiate myoblasts from fibroblasts using labelled antibodies (Skuba, 1969, Rukossuev, 1969).

Thus we can see that a number of investigators reported that regeneration of heart muscle fibers could be obtained in adult mammals under certain conditions.

On the other hand, as has already been noted (Mirakian and Rumjantsev, 1968; Sarkisov, 1968), some authors continue to dispute the possibility of heart muscle fiber regeneration in adult mammals. These authors suggest that if the ability for DNA synthesis in heart muscle nuclei is lost (repressed) as well as the ability to the mitotic division, the regeneration of muscle fibers is impossible.

They do not take into account that regeneration might occur by other, so far unknown, means, for example, by migration of myogenic elements into the granulations with their subsequent activation (or induction) by heart muscle degradation products involving derepression of DNA synthesis and recovery of proliferative activity. Sinitsyn (1969) stressed that in his experiments with dogs, myoblasts were formed by the migration of cells separating from the stumps of papillary and trabecular muscle fibers. This takes

place a month after the operation. The mechanism of myocardium regeneration is very obscure and demands further studies.

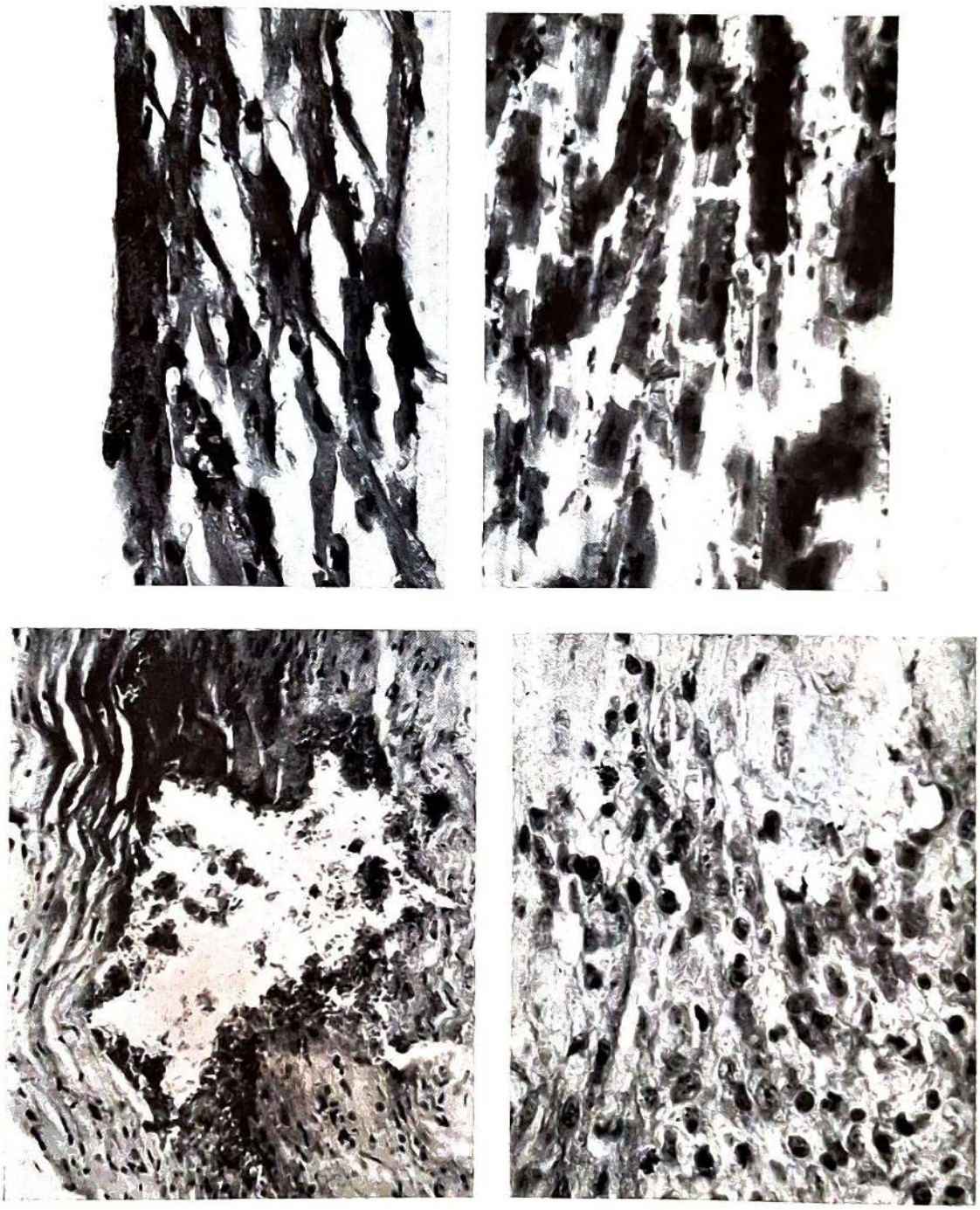

Figure VI-27. Changes in the rabbit heart under the action of diptheritic toxin. Ocular 7, objective 60. Top left, Hyaline degeneration of heart muscle fibers. Top right, Lumpy disintegration of muscle fibers. Bottom left, Foci of disintegration of muscle fibers. Bottom right, Scar tissue arized in the focus of disintegration of muscle fibers.

PROBLEMS AND PERSPECTIVES

In our laboratory we began studies on stimulation of heart muscle regeneration after diphtheria induced myocarditis in rabbits (Polezhaev *et al.*, 1965, 1966, 1967, 1969; Kolchin, 1966, 1967, 1967a; Sadokova, 1966, 1967, 1968; Latysheva, 1968; Maliovanova, 1968). Administration of diphtheria toxin in certain doses leads to the development of diphtheria myocarditis in 100 per cent of the cases. This was accompanied by degeneration and degradation of muscle fibers, no regeneration was noted (Fig. VI-27). The injection of heart muscle hydrolysate or other preparations (RNA vitamin B_{12} etc.) to the experimental rabbits improved certain morphological, biochemical and physiological parameters. The formation of myoblasts and young muscle fibers was also noted (Fig. VI-28, 29).

Another important task is the study of regenerative processes in myocardium at the cellular, submicroscopical and molecular level. This necessitates application of a number of modern methods such as electron microscopy, autoradiography, cytospectrophotometry, etc.

Another interesting direction is heart muscle regeneration after the infarction in humans. For this purpose the region damaged by the infarction must be removed, and this is already attained in experiments with dogs (Murray, 1947, Carter and MacMillan, 1950; Sergievsky, 1958). After the removal of the infarcture a patch must be applied according to the method of Sinitsyn. When such operations are conducted with dogs, the lethality does not exceed 5 per cent and heart muscle regeneration is observed in 100 per cent cases. The size of regenerated muscle may be different but it can be increased by the application of regeneration stimulants. This is much more simple and safe than heart transplantation, which is successfully conducted at present in humans. Tissue incompatibility needs not be circumvented. There are reasons to believe, that this approach is quite realistic and may be interesting for medicine.

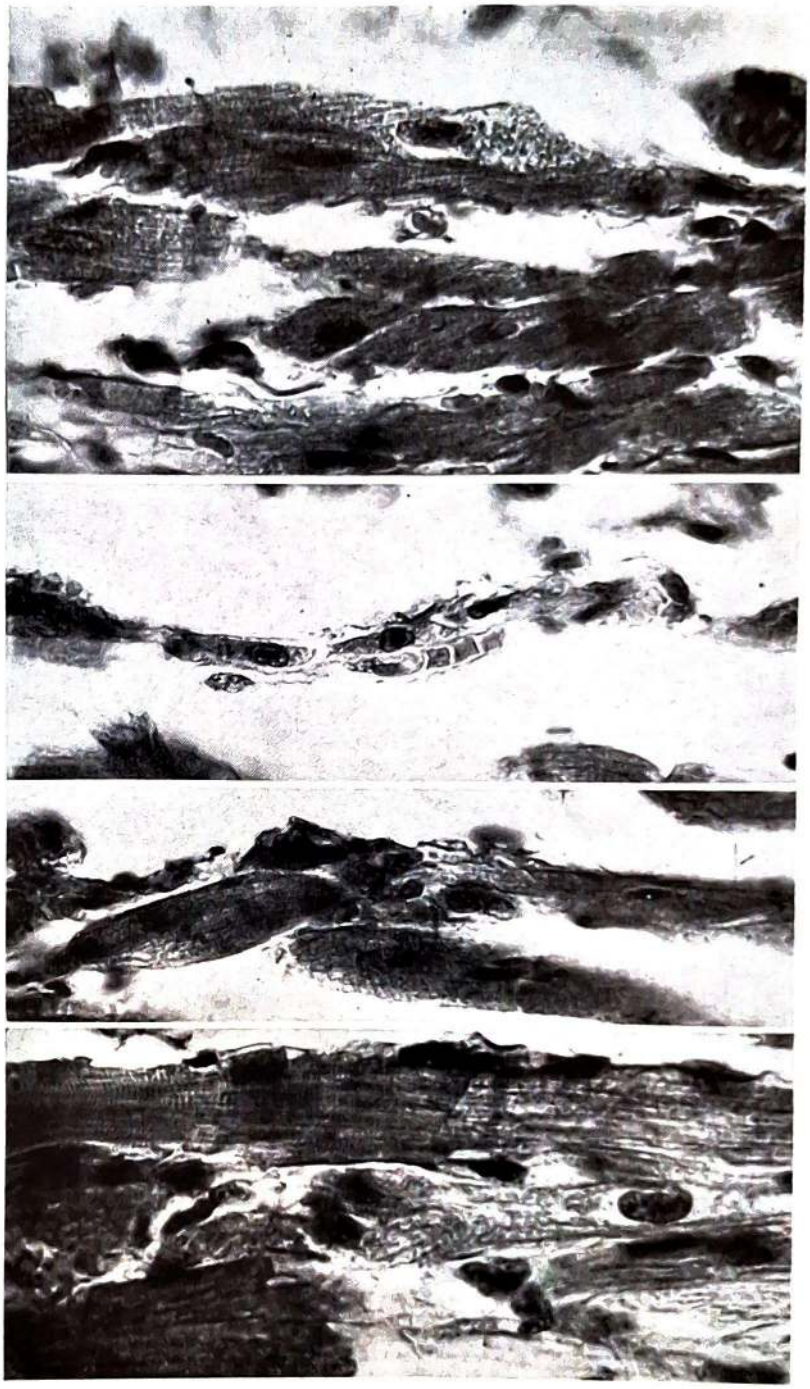

Figure VI-28. Stimulation of regeneration in cardiac muscle under diptheritic myocarditis in rabbit. An experiment with hydrolysate of myocardium. Ocular 7, objective 60. Top, Dedifferentiated muscle fiber stumps; Second, Chains of myoblasts between muscle fiber stumps; Third and bottom, Thin muscle fibers of young type among degenerated muscle fibers.

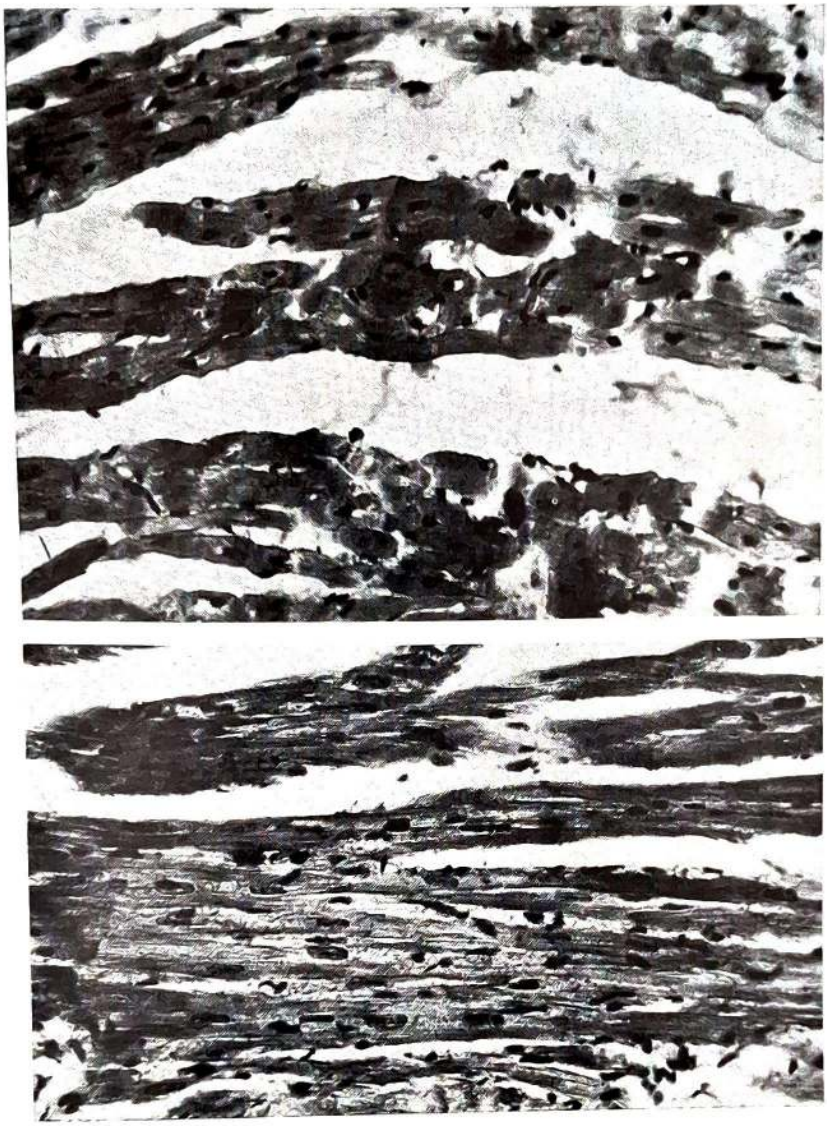

Figure VI-29. Stimulation of regeneration in rabbit heart muscle under diphtheritic myocarditis. Ocular 10, objective 20. Top, An experiment with toxin alone, 120 days after administration of toxin. Edema, degeneration of muscle fibers. Bottom, An experiment with toxin and administration of preparations. 120 days after administration of toxin and 15 and 45 days after administration of hydrolysate of myocardium, RNA and vitamin B_{12}. Edema disappears, general structure of muscle fibers is normalized.

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VII

STIMULATION OF NERVOUS TISSUE REGENERATION

FORMULATION OF THE PROBLEM

Is the regeneration of brain nervous tissue in mammals and man possible? To answer this question we shall consider the available literature and then our own experimental results.

The problem of nervous tissue regeneration in animals and man is extensively reviewed in a number of surveys (Marchand, 1901); Goldziher and Makai, 1913; Cajal, 1928; Spielmeyer, 1929; Boecke, 1935; Windle, 1955; Kirsche, 1965; Murray, 1965; Polezhaev, 1968 and others).

Up to very recent time the textbooks of histology and embryology stated that the brain and spinal cord in mammals and man were unable to regenerate (Zavarzin and Rumjantsev, 1946; Zavarsin and Shchelkunov, 1954; Knorre, 1959 and others). Nervous tissue of the brain and spinal cord in these organisms is formed predominantly during embryogenesis (His, 1880-1885, 1893). During this stage neuroblasts form neurons which lose the ability for mitotic division during postnatal life and cannot divide amitotically. In the postnatal period animals do not possess undifferentiated cells of the cambial type which might give rise to neurons. Spongioblasts form glial cells, which retain the ability to proliferation even in the postnatal period of ontogenic development. On the basis of these data most histologists and embryologists came to the conclusion that nervous tissue was stable and incapable either to physiological or repair regeneration. The number of neurons in adult animals or humans equals their number in the brain at the end of embryogenesis; possibly they somewhat decrease in number due to degeneration and degradation with age. Only peripheral nerves (that is the processes of nerve cells) are able to regenerate under certain conditions, while neurons themselves cannot regen-

erate. At the same time there are results in the literature which demonstrate that in certain species of lower animals not only division or *de novo* formation of nerve cells is possible, but also reparative regeneration of considerable parts of the central nervous system. Moreover, stimulation of nervous tissue regeneration is possible.

STIMULATION OF REGENERATION IN THE PERIPHERAL NERVOUS SYSTEM

The regeneration of nerve stems and nerve endings, representing the processes of the nerve cells is a long-known fact. More than that, the rate of peripheral nervous system regeneration in man is known. It corresponds to about 1 to 2 mm per day. Peripheral nerves regenerate in all vertebrate animals. A number of methods for the stimulation of such regeneration of nerve stems was suggested—administration of vitamins B_{12}, thyroid hormone, high temperature and so on (Znachkova, 1957). The presence of distal degenerated nerve terminus is very essential for the regenerative process. It is a good conductor for the growing axons, it contains neurotropic agents, stimulating the growth of nerve endings, and it contains Schwann cells (cells of connective tissue) which support the growth of these latter. This is why nerve homotransplantation is used sometimes for the recovery of the damaged nerve. Anokhin (1944) suggested the heterotransplantation of formaline-treated calf nerve to human patients. He obtained favorable results; the nerve regenerated, grew through the transplant and the function of the extremity was recovered (Anokhina, 1954). Efimov (1966) performed homotransplantation of three fresh *n. ischiaticus* on the place of almost completely removed *n. ischiaticus* in rabbits. During two weeks after the operation the animals were subjected to hypothermy and brought into sleep using urethane. Under these conditions the nerve regenerated and function of the extremities was regained. Regeneration of nerves depends on their ability to destruction; this was shown in experiments with rats and rabbits using methods of neurohistology and biochemistry. After the cutting of the optical nerves (n. opticus) they show weak destruction and do not regenerate; while in peripheral nerves such as *n. ischiadicus* and *n. tibialis,* the destroyed processes are strong and regeneration is also very good (Nesmeyanova, Gutmann and Haek, 1966).

Interesting results on stimulation of peripheral nerve regeneration were obtained in the laboratory of Muralt (Jent, Koechlin, Muralt and Wagner-Yauregg, 1945; Muralt, 1946; Lüscher and Muralt, 1947; Koechlin and Muralt, 1947; Koechlin, 1955). These authors isolated a neuroregenerative substance, called by them NR, which stimulated the regeneration of peripheral nerves. It was obtained both from brain and spinal cord or from peripheral nerves. They used a convenient test: rabbit cornea, the nerves of which were cut in the shape of arch (Jent, 1945). It was found that the injection of NR leads to fourfold to fivefold stimulation of the regeneration of these latter. NR is a substance of a nonprotein nature, it is found in the dialysis fluid after the dialysis of the extract; it is heat labile and most active in the concentration 6×10^{-5}/ml.

A series of very interesting investigations was conducted by Levi-Montalchini and co-workers during the last fifteen to twenty years (Levi-Montalcini, 1952, 1955, 1965, 1965a, 1966). It was shown that the growth of processes of nerve cells during the cultivation of neurons from the sensory and sympathetic ganglia of chick embryo *in vitro* was considerably stimulated by the addition of the nucleoprotein extract from mouse sarcoma, snake venom, protein extract from the maxillary salivary glands of the mouse or from the sensory or sympathetic ganglia of rats, mice, cats, cow or man. These latter extracts stimulate only the ganglia from the sympathetic nervous system in chick embryo *in vitro* or in mice *in vivo*. Application of these extracts lead to the increase of the volume of sympathetic chains ganglia in newborn mice about fivefold and in adult mice about twofold. The growth of dendrites and neuron bodies is stimulated, their hypertrophy is observed. The nerve growth factor is of protein nature.

These results were confirmed in other laboratories in experiments with mice (Rodionov, Jarygin, Kuzmicheva *et al.*, 1968).

STIMULATION OF THE SPINAL CORD REGENERATION

When the spinal cord in fishes, larvae and adult urodeles is cut, it undergoes efficient regeneration, leading to the recovery of the damaged function; the ependyme cells of the spinal cord channel serve as a source of its regeneration (Piatt, 1955, 1955a; Koppanyi, 1955; Kirsche, 1956, 1965; Winkelmann, 1963; Butler and

Ward, 1965 etc.) It is also known that tail removal in amphibia leads to the regeneration of this organ, and after the regeneration it contains the spinal cord. Regeneration of the tail and spinal cord in axolotles can be stimulated when the experimental animals are fed with the embryonic pig liver (Richter, 1966). The ability to regeneration in reptiles and birds is considerably lower (Hamburger, 1955).

In mammals and man the spinal cord does not regenerate after the complete cutting or rupture; this leads to paralysis (Windle, 1955). It should be noted however that application of pyromen, cortisone, ACTH, desoxycorticosteron acetate or trypsin leads to certain recovery of the damaged function and some regeneration of the spinal cord axons in rats, cats and dogs (Clement, 1955; Scott, 1955; Stewart, 1955; Turbes and Freeman, 1953; Freeman *et al.*, 1960). Pyrogenal and other mentioned preparations lead to such alteration of the neuroglial scar, that some regenerating axons of the spinal cord could grow through it. These preparations abolished the strong pressure developed by the scar, which prevented regeneration and the survival of axons in the damaged region.

Nesmeyanova (1968, 1968a) and Nesmeyanova *et al.* (1960, 1961, 1963) cut the spinal cord in dogs and rats and inhibited the formation of the neuroglial scar by pyrogenal and trypsin; on the other hand they stimulated the regeneration of spinal cord axons by the transplantation of fresh or formalin-treated pieces of the spinal cord into the damaged region. Another procedure consisted of administration of malonic acid dinitryl (MADN) to the experimental animals. This drug increases RNA content in neurons (Hyden, 1947). The authors were able to recover the locomotory function in dogs, which lived for 2.5 years after the operation. Axon regeneration was evident by morphological criteria. The best results were obtained with MADN and pyrogenal. Trankvilitaty and Nesmeyanova (1961) used pyrogenal therapy for the treatment of eight patients with complete or partial rupture of the spinal cord. The course of treatment lasted for two to four months. Partial recovery of locomotory function of the legs was noted in all patients. It was more complete in persons with partial rupture of the spinal cord. This was accompanied by the increase in mass of muscles and restoration of the sex function. Pyrogenal therapy permitted the recovery of functions in patients and in children with

damaged spinal cord as reported by a number of other authors (Kogan, 1965; Grishukova, 1965).

REGENERATION OF BRAIN NERVOUS TISSUE

Before turning our attention to the vertebrates, we shall briefly discuss the regeneration of nervous element invertebrate animals. In some invertebrate species the regeneration of nerve cells is possible after complete or partial destruction of their nerve ganglia. Belova (1968) has shown in a comparative investigation that "brain" removal in *Planaria lugubris* leads to its complete regeneration within the next ten days. In rain forms *Allobophora longa* and *Eisenia foetida* the regeneration takes forty days. In the leech *Haemopis sanguisuga* the subgullet ganglion regenerated completely within thirty days after partial destruction. After the complete removal the atypical regeneration of the ganglial mass was observed. In river crayfish (*Astacus astacus*) and black beetle (*Blatta orientalis*) regeneration of the ganglia after the partial destruction occurred within twenty-five days. According to Belova the regeneration of ganglia in all these invertebrate species is due to proliferation and differentiation of weakly differentiated reserve cells; in addition it occurs through the mitotic and amitotic divisions of differentiated nerve cells. Similar observations about the neural ganglia regeneration in invertebrates were reported by other authors (for literature see Belova, 1968).

The problem of nerve cell regeneration in the nervous system of vertebrates, mammals in particular, is especially difficult and complex. This is due to several factors. It has already been mentioned that nervous tissue in these organisms is static—neurons do not proliferate and are highly specialized in the postnatal period of ontogenic development. This is shown by results of both embryology and histology. The nuclei of neurons do not synthesize RNA (Leblond, Messieur, Kopriwa, 1959; Leblond, 1964). Not only neuron proliferation but also simple growth of nervous tissue *in vitro* was an impossible task for a long period of time (Rumyantsev, 1932; Murray, 1965). Results of clinical and patoanatomical investigation demonstrated the irreversible changes in the damaged brain nerve tissue in mammals and man (Marchand, 1901; Goldzieher and Makai, 1913 *et al.*). Nevertheless the literature contains the data showing that the problem of nerve cell proliferation and

nerve tissue regeneration in vertebrates is not completely hopeless.

It was found that regeneration of relatively large brain regions in fishes and amphibia was possible. The regeneration of tectum opticum in fishes such as *Carassius carassius* and *Lebistes reticulatus* was demonstrated (Kirsche, 1959, 1960, 1965; Richter, 1966, 1968). Regeneration of telencephalon was reported in *Gastrosteus aculeatus L.* (Segaar, 1962, 1965) and *reticulatus* (Maron, 1963). The source of regeneration is represented by the cells of ependyme or matrix (Kirsche, 1963). At the same time Pflugfelder (1964, 1965) failed to observe the regeneration of telencephalon and mesencephalon in *Carassius gibelio auratus* and *reticulatus*.

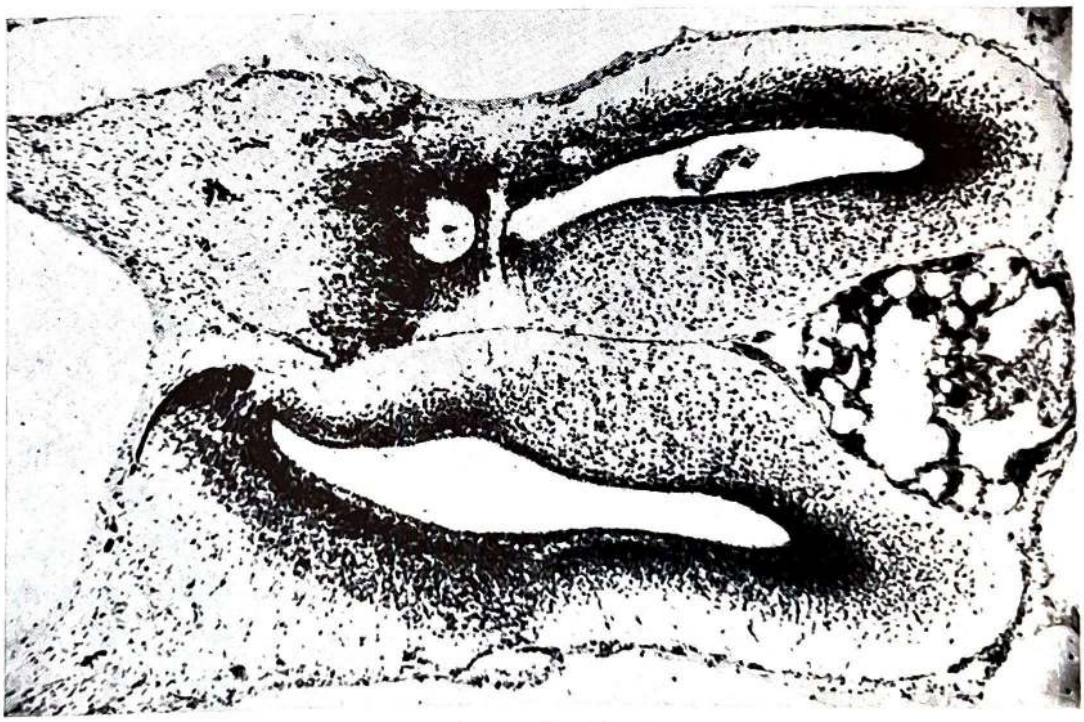


Figure VII-1. Regeneration of anterior brain with additional ventriculum (top), 150 days after operation in axolotl; on the bottom is nonperated anterior brain, x 160 (Kirsche, 1964).

The regeneration of different parts of brain and cerebellum was shown in experiments conducted with tadpoles, adult frogs, axolotles, and newts of different age (Danilewsky, 1891; Weissfeiler, 1924, 1925; Sibbing, 1954; Srebro, 1957; Jordan, 1958; Kosciuszko, 1958; Kwiatkowski, 1961; Kirsche, 1964, 1964a; Polezhaev and Maliovanova, 1965; Maliovanova and Polezhaeva, 1966; Filoni, 1968, 1968a; Richter, 1968, 1968a) (Fig. VII-1,2,3)

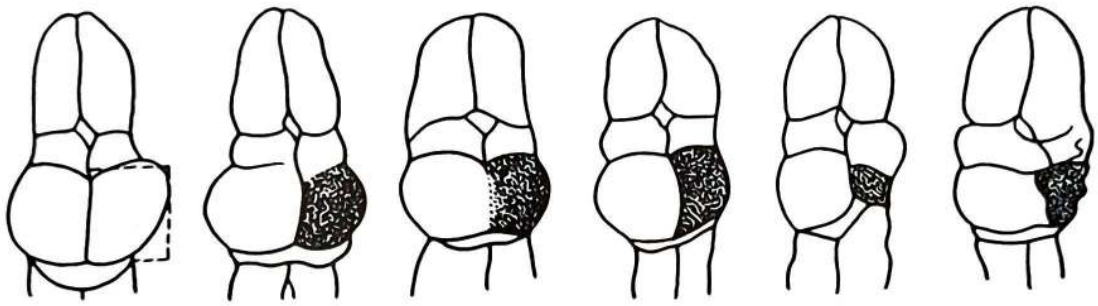


Figure VII-2. Regeneration of tectum opticum in tadpoles *Rana temporaria* after its removal from the right side at the stage Ic; on the left is nonoperated side of brain. Far left, broken line indicates the zone of extirpation of brain (it is dark) thirty days after its removal.

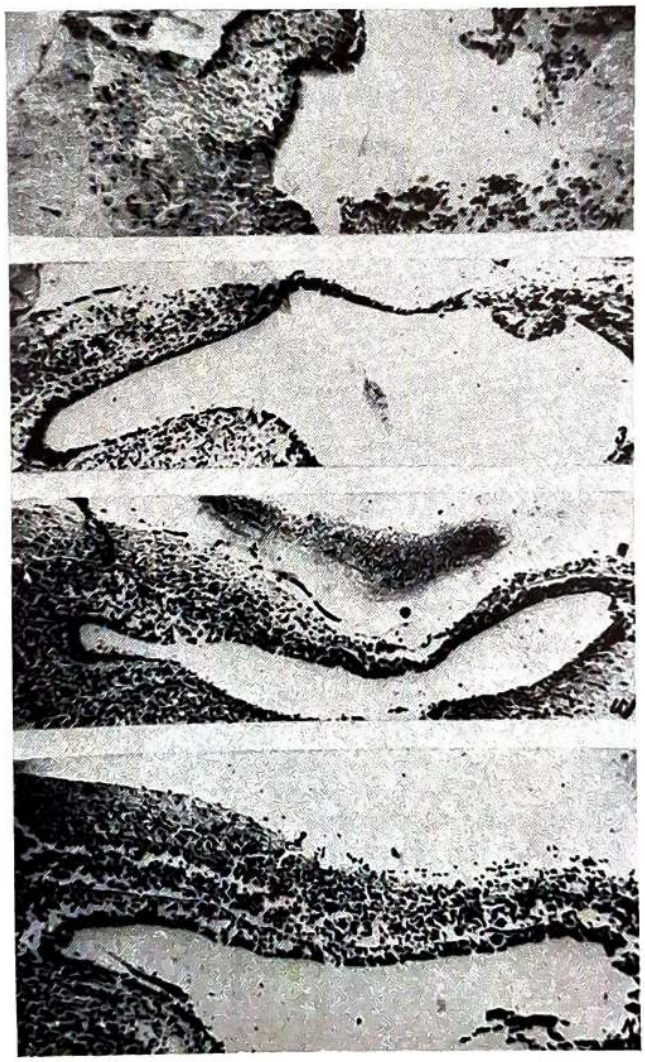


Figure VII-3. Histological vertical sections of regenerating tectum opticum in tadpoles of *Rana temporaria*. Successive stages of regeneration. On the right is the zone of regeneration and on the left, nonoperated brain.

Kirsche (1967, 1968) notes that regeneration of the brain in amphibia, reptiles, birds and mammals may occur at the expense of postnatal matrix zones corresponding in their localization to embryonic matrix zones. These zones consist of small basophilic undifferentiated cells located in many layers. After intensive mitotic proliferation or even in the absence of proliferation, they can be converted into differentiated nervous and glial cells building the regenerated part of the brain.

Injection of extracts from the embryonic pig brain to the axolotles with a removed part of telencephalon stimulated the regeneration and increased the number of nerve cells in the regenerate (Kirsche, 1965) (Fig. VII-4).

Turning to mammals we must mention several groups of facts. Experiments on cortex removal in two to four-week-old kittens or puppies created an impression of anatomical recovery of the nervous tissue in the damaged region (Kogan and Ivannikova, 1955; Dzidzishwili, 1956; Mepisashvili, 1960; Polezhaev, Matveeva and Zakharova, 1957). Further experimental and histological studies, however, demonstrated that the observed effects are due not to regeneration but to the displacement of the nervous tissue from the adjacent regions to the damaged zone (Klossovsky and Vasiliev, 1961; Ivannikova, 1963; Belova, 1964; Polezhaev and Resnikov, 1966; Wenzel *et al.*, 1969, 1969a). The frequency of neuron amitotic divisions become increased in the remaining part of the cortex and abortive mitoses are visible in the border zone. Although the cortex remains damaged, the condition reflexes immediately after the operation, this function is recovered in a relatively fast manner, in contrast to the situation with the adult animals of the same species (Kogan and Ivannikova, 1955; Ivannikova, 1960).

On the other hand it should be noted that damage of brain cortex, cerebellum or other parts of the brain by ionizing radiation at the embryonic or early postnatal stage in mice, rats, rabbits, guinea pigs and monkeys leads to the death of a part of neuroblasts and nerve cells. They are substituted by new cells formed by the mitotic divisions of the remaining ones (Manina, 1964). The author concludes that at these stages of ontogenic development the nerve tissue in these mammalian species retains the ability to physiological regeneration. Altman, Anderson and Wright (1969) irradiated

Figure VII-4. Stimulation of regeneration of telencephalon in axolotl after removal of its right anterior third and administration of extract of brain of pig embryos. Three hundred days after operation (Kirsche, 1965). Top, Rear terminal pole of brain without stimulation (control); Bottom, Rear terminal pole of brain with stimulation; increase in the number of nerve cells.

heads of young rats by small doses of x-rays and observed degradation of the external granular layer of the cerebellum which was followed by its rapid recovery (Fig. VII-5). A similar phenomenon was observed by Antonova (1968), who irradiated cerebellum of adult guinea pigs with x-ray doses of 9000 rad.

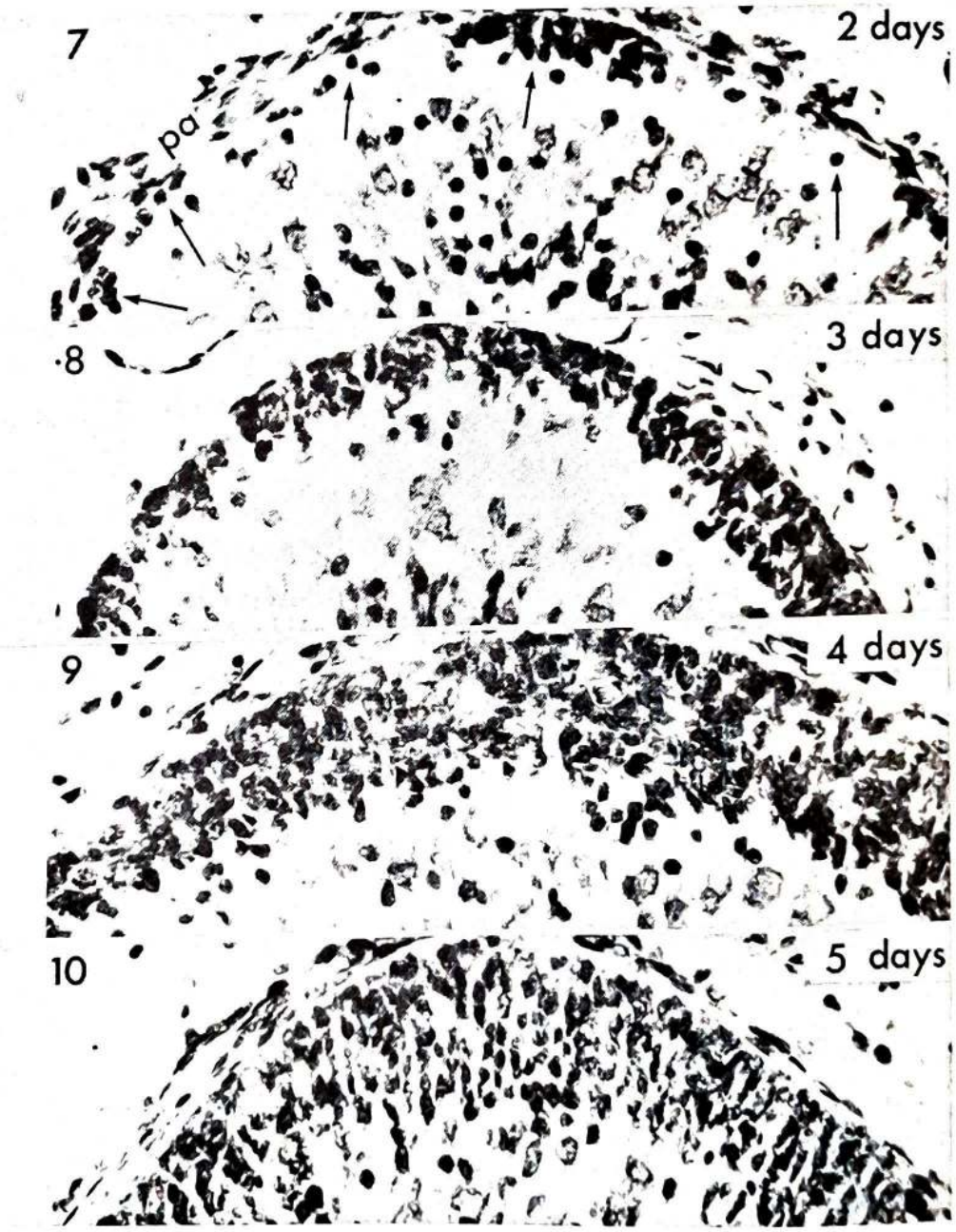


Figure VII-5. Photomicrographs of the external granular layer over the lobus centralis ventralis in animals exposed to a single dose of 200 x-ray, with survival after irradiation ranging from two to five days. Arrows in part 7 point surviving isolated cells or cell clusters representing the decimated external granular layer; pa, pia-arachnoid membrane. Cresyl violet, x 400 (Altman, Anderson and Wright, 1969).

The problem of the nerve cell's *ability* to divide was reinvestigated and vividly discussed in the literature. Several authors reported the amitotic division of neurons in sympathetic ganglia and ganglia of certain internal organs in rabbits, rats, dogs, cats, horses and man. There is also literature concerning the cortex, retina, cerebellum and other brain regions. The results concern normal and pathological conditions; they were obtained with young and adult humans, mammals (monkeys, dogs, rabbits, rams, goats, wolves and rats) and birds (for literature see Vasile, Hudruc and Gheorghiu, 1961; Kirsche, 1965; Polezhaev, 1968). A number of authors described consecutive stages of neuron amitotic divisions; stage of two nucleoli, two nuclei and two-cellular (cytotomy) (Popoff, 1875; and a number of recent authors e.g. Afanasiev and Kotovsky, 1959, 1961; Krivitskaya, 1964; Rampan, 1956, 1961; Belova, 1964, 1968; see Kirsche, 1965; Vasile, Hudruc and Gheorghiu, 1961; Polezhaev, 1968). Torskaya (1963) reported that amitotic division of the neurons began with the formation of furrow in the nucleus. She was working with the brain cortex and nuclei of diencephalon, mesencephalon and spinal cord of adult dogs subjected to reanimation after the clinical death.

Several investigators described mitotic division of neurons near the damaged regions in the cortex of large hemispheres or rats or rabbits brains (Kirsche, 1965; Polezhaev, 1968). It was noted, however, that neuron mitoses were abortive: prophases and metaphases were seen while anaphases and telophases were not observed; this was due to the atrophy of dividing neurons (Soskin, 1965, Wenzel *et al.*, 1969, 1969a).

The nervous tissue of the human or animal (rabbit, dog, monkey) brain can be cultivated *in vitro* for as long as several months using the improved methods (Hogue, 1947; Costero and Pomerat, 1951; Geiger, 1957, 1958, 1963; Mastrogiovany, 1958; Murray, 1965; May, 1966). The time of nervous tissue survival in culture *in vitro* can be considerably prolonged by the addition of brain extracts to the culture medium. The explanted neurons are capable of mitotic division, cortisone addition stimulates their number at least eightfold; the formation of binuclear neurons was also observed (Geiger, 1957, 1963). Mitotic divisions of neurons in the nerve tissue culture was also described by other investigators (Levi and Meyer, 1933; Murray and Stout, 1947).

There are indications that the nervous system of mammals during the postanal period of their life contains neuroblasts which can be converted into differentiated neurons (Potapova, 1957; Coujard and Mailett, 1960; Vasile, Hudruc, Gheorghiu, 1961, Torskaya, 1963, 1964).

Criticizing the concept of neuron ability for division, Jabotinsky (1958) and Windle (1955) suggest that glial cells and macrophages may be erratically taken for dividing neurons; the neuron nucleus may divide, but not its body, this may lead to the formation of the binuclear neurons. These possibilities are quite realistic, but we do not think that all the facts of neuron division described in the literature can be explained by them.

The problem of amitotic division in general and in application to the nerve cells has been subjected to revision during the last few years (Bucher, 1959; Brodsky, 1966 etc.). Without dwelling too much upon this very complex problem we shall make only a few remarks. The possibility of amitotic division of nuclei in different cells is no longer disputed. Probably it occurs most often by lacing of the polyploid nucleus, after the replication of its DNA. Amitotic division of skeletal muscle fiber nuclei was demonstrated by time-lapse microcinematography (Pûzá, 1966). The possibility of cytotomy is disputed by a number of authors, but nobody proved that it is impossible. In tissue culture *in vitro* amitotic division was also traced by time-lapse microcinematography (Boll, Kretschmer, Fliedner, 1967). It can be suggested that DNA synthesis or possibly endomitosis (Geitler, 1963) preceded the amitotic neuron division. It is not impossible that this endomitosis ended with the lacing of the nucleus and the cytoplasm. The latter was also described for the cortex of large hemispheres in several mammalian species (see above). A reservation should be made, however, that this complex problem demands further studies with autoradiographical, cytospectrophotometrical and cinematographical methods. Proliferative inactivity of neurons (and muscle elements) in the adult organism was demonstrated by autoradiography with thymidine-H^3. No DNA synthesis is observed in their nuclei and they do not divide (Leblond, Messieur, Kopriwa, 1959). The same method was applied to the brain of mice, rats and cats and it was shown that the matrix zone was located in the region of lateral ven

tricles; this zone consisted of ependyma and multi-layered subpendymal accumulation, which contained weakly differentiated cells (Sidman, Miale and Feder, 1959; Angevine and Sidman, 1961 etc.). These cells intensively synthesized DNA, underwent mitotic divisions and migrated through the white matter into the grey one to the surface of the large hemispheres. A part of these cells was converted into glial cells (Smart and Leblond, 1961; Smart, 1961). Other cells were converted into neurons (Fig. VII-6,7) (Altman, 1962, 1963; Altman and Das, 1965, 1965a, 1966, 1966a). It can thus be concluded that in rats and some other mammalian species neurons of the hemisphere cortex during the postnatal ontogenesis can be derived from weakly differentiated cells of cambial type, in subependymal or matrix layer. In the cerebellum neurons and glial cells differentiate from cells of external granular layer, which plays the role of external matrix (Miale and Sidman, 1961; Fujita, Shimada and Nakamura, 1966 etc. for literature see also Manina, 1964; Gracheva, 1968). Thus we witness definite revision of our concepts about brain development and origin of the nerve cells, the concepts laid down by His (1880, 1893). It might be that not only glial cells, but also a certain amount of neurons are permanently renewed at some stage of the postnatal development or even in some adult mammals. It cannot be excluded that similar phenomena take place in humans. The

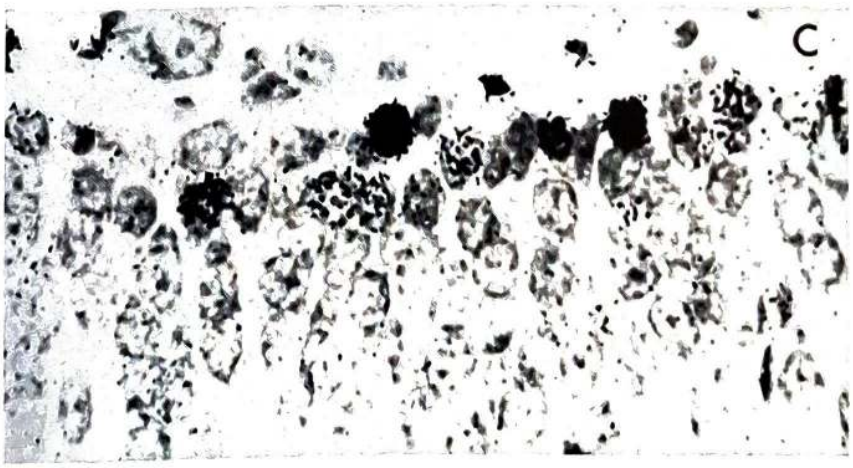


Figure VII-6. Low and high power microphotographs of autoradiograms from the area of the dentate gyrus of the hippocampus in a rat injected with thymidine-H^3 at the age of ten days and killed two months after the injection. Note labelling of granule cells, predominantly in the internal border (basal surface) of the granular layer, x 640 (Altman and Das, 1965).

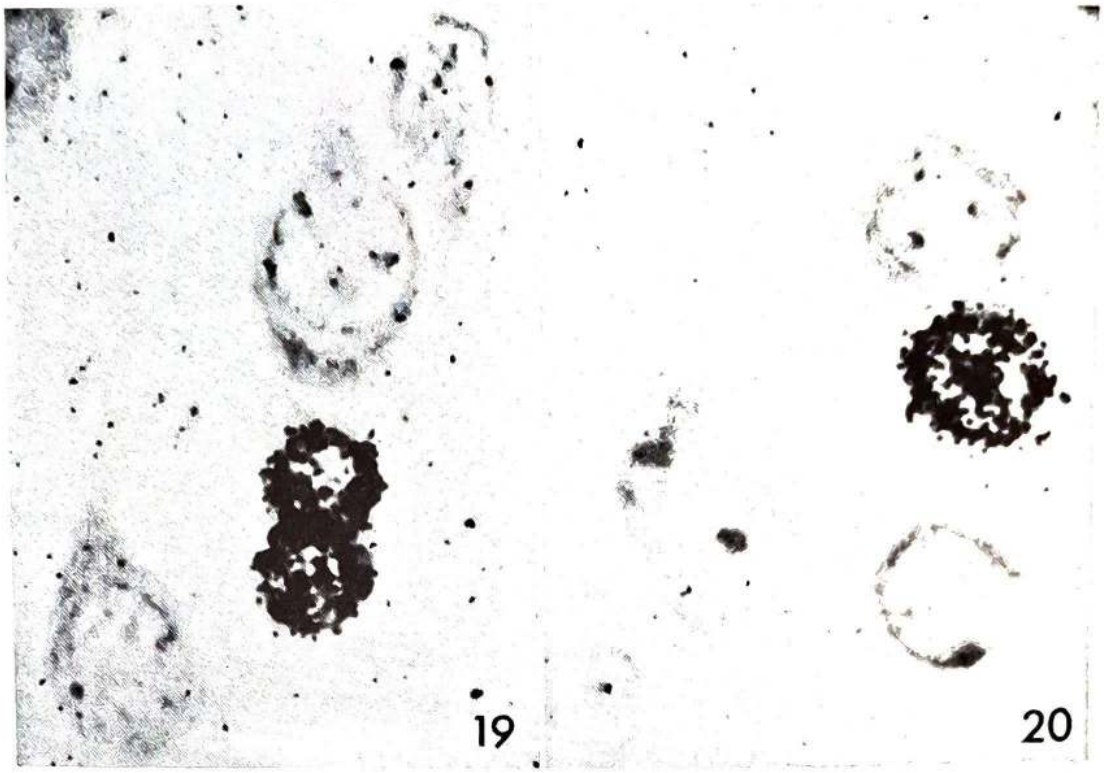


Figure VII-7. High-power photomicrographs of well-labelled cells from the cortex of an animal injected at six days of age and killed twenty days later. The cells shown in 19 may be small neurons; this type of cells is often seen labelled. The type of labelled larger neuron shown in 20 is only occasionally encountered. Gallocyanin chromalum. x 640 (Altman, 1966).

problem of cell population renewal or physiological regeneration of brain tissue presents considerable interest for biology and medicine. Can this population renewal and regeneration be stimulated?

PHYSIOLOGICAL REGENERATION STIMULATION OF BRAIN NERVOUS TISSUE

In our laboratory we made an attempt to stimulate physiological regeneration of the cortex nerve tissue in adult mammals (Polezhaev, 1961, 1968; Polezhaev and Karnaukhova, 1962, 1963; Reznikov, 1965, 1966, 1967, 1968; Reznikov and Kolchin, 1966; Reznikov and Kondik, 1966). We suggested that the molecular and cellular renewal of the brain nerve tissue can lead to the alteration and improvement of the function of the central nervous system of the animal. The achievement of this goal would give us a new biological method for the therapy of certain diseases of the central nervous systems or secondary diseases, caused by its abnormal state. The task, therefore, was to stimulate destruction and dedifferentiation of the nervous tissue in the brain cortex.

To evoke these phenomena a number of procedures were employed. Their choice was based on the following two principles: a) the existence of different physiological stages in regeneration and b) the principle of organ specificity. For this purpose pieces of brain were homotransplanted into the right hemisphere of the brain in adult rats or mice. In other experiments pieces of agar, brain homogenates, dialysates (dialysis against distilled water was conducted) high polymer RNA from rat liver or physiological saline were administered. Some of these preparations were injected into the liquor of the fourth ventricle or given subcutaneously in order to elucidate the role played by the hematoencephalic barrier. The results were evaluated by histological, neurohistological, histochemical, autoradiographical and cytophotometrical methods. The data obtained were subjected to statistical analysis. In physiological experiments which were conducted on the basis of Schoen method (Schoen, 1926), the balance of inhibitory and excitatory processes dependent on the state of the cortex and subcortical layers was studied. Our main results can be briefly summarized as follows.

In different series of the experiments the results were on the whole similar, but they differed in the degree of alterations in the nerve tissue of the cortex. Most of the expressed changes were caused by the implantation of brain pieces into the brain, this was

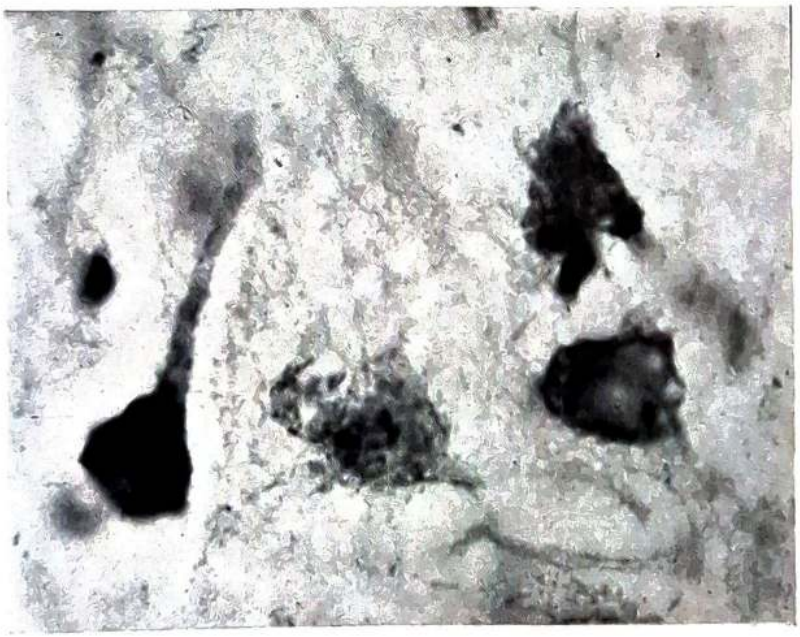


Figure VII-8. Degeneration and death of neurons. Thirteen days after operation of brain in rats. Ocular 7, objective 90.

followed in the sequence of decreasing effect by the injection of brain homogenates, brain dialysates in the final concentration of 6×10^{-5} g/ml, RNA and the smallest effect was observed after the saline injection. Besides general phenomena associated with the aseptical inflammation, which developed within a few days after the administration, alterations in the cortex nerve tissue were recorded. On the one hand, atrophy of neurons, accompanied by their swelling and wrinkling, was increased (Fig. VII-8). On the

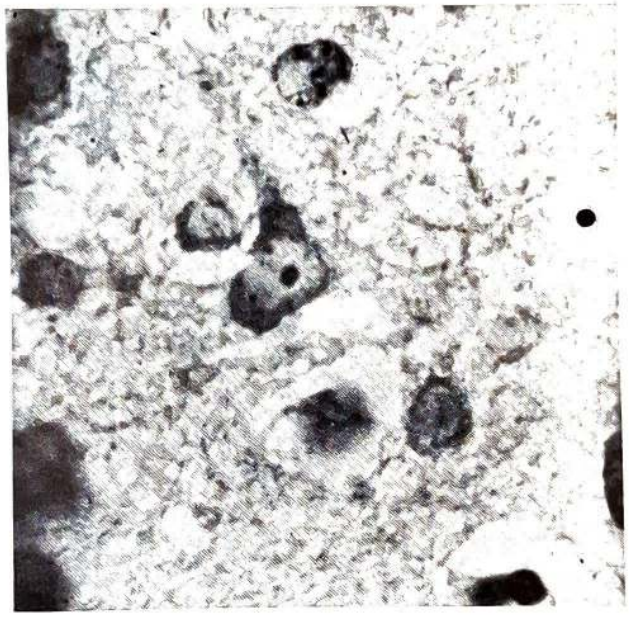


Figure VII-9. Formation of young neurons in place of dead ones in the periphery of the focus of injury in cerebral hemispheres in a rat. Thirteen days after operation. Ocular 7, objective 90.

other hand, formation of neurons *de novo*, possibly from subependymal cells, was observed (Fig. VII-9) as well as stimulation of neuron amitotic division (Fig. VII-10). These amitotic divisions were preceded by changes, which could be analogous to dedifferentiation. The Nissl substance disappeared from the neuron cytoplasm, the nucleus became large and clear and the nucleolus also increased in size (Fig. VII-10,b). Then the nucleolus divided, newly formed nucleoli migrated to the opposite poles of the nucleus, the nuclear substance concentrated around them, the nucleus was laced and this was followed in some cases by the division of the cell body. Binucleolar, binuclear and bicellular nerve cells were formed. Sometimes the nerve cells were located in groups of two (Fig. VII-10,c-h). In some cases neurons divided a second time

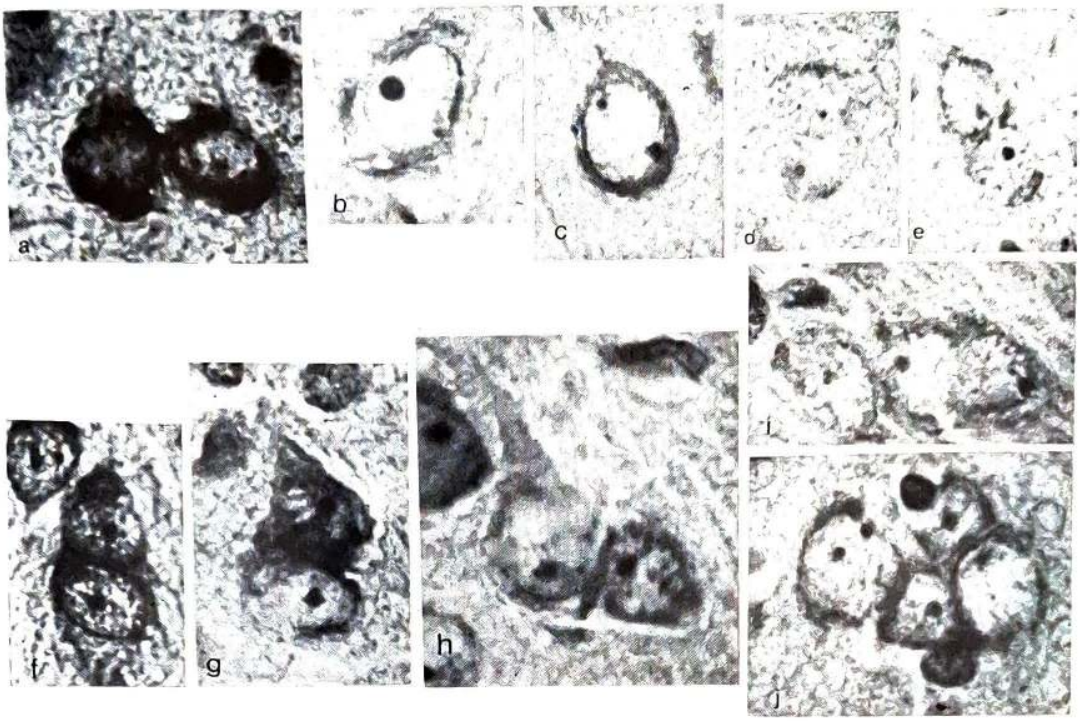


Figure VII-10. Successive stages of amitotic division of neurons in the cortex of cerebral hemispheres under stimulation of physiological regeneration of nervous tissue of brain in rat. Ocular 10, objective 90. a-neurons in the control; b-j-neurons one day after grafting a piece of agar into the brain; b,c-binucleolar stage of amitosis of neurons; d,e,f,g-binuclear stage of amitosis of neurons; h-division of neuron body; i,j-division of neurons into 3 and 4 cells.

this resulted in the formation of clusters, consisting of three or four neurons (Fig. VII-10, i,j).

The number of binucleolar and binuclear neurons as well as of those that were located pairwise was maximal at the third to fifth day after the administration of preparations into the brain and returned to the normal level ten to seventeen days later (Fig. VII-11, 12).

Repeated administration of these preparations into the brain permitted to prolong the process of neurons amitotic division. The changes in the nerve tissue of the brain cortex were observed when the preparations were injected directly into the brain or into the liquor of the fourth ventricle they were not observed after the subcutaneous administration.

It should be emphasized that every binucleolar neuron could not be coverted into the binuclear one and every binuclear neuron could not divide into two cells.

Neuron mitotic division was observed only in experiments with

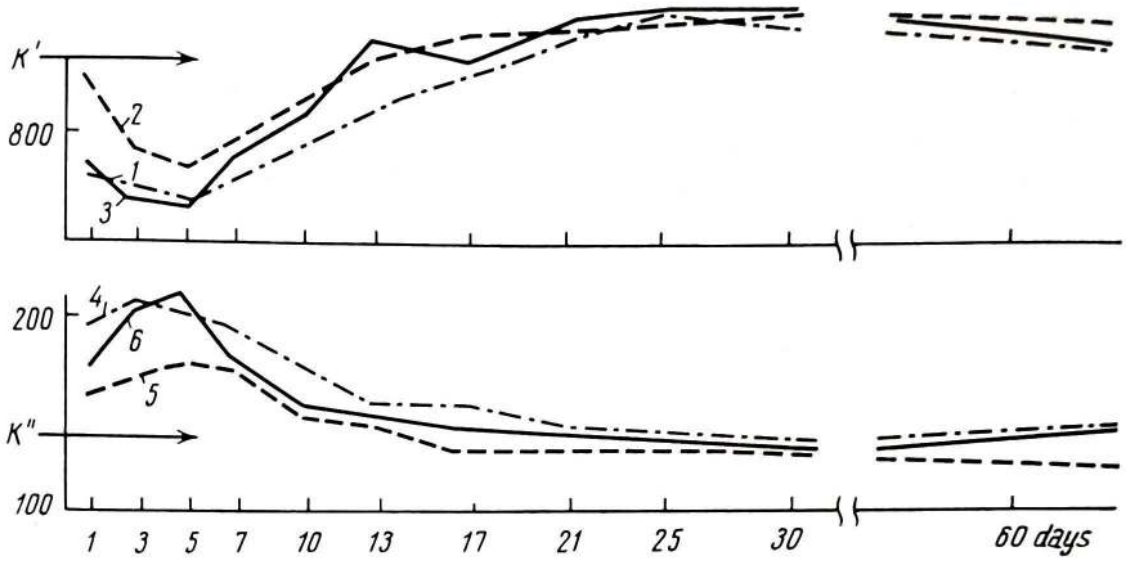


Figure VII-11. Dynamics of changes of the number of mononucleolar and binucleolar nerve cells after administration of biostimulators into the brain. 1-the number of mononucleolar cells in experiment with implantation of a piece of brain: 2-the number of mononucleolar cells in experiment with administration of brain homogenate; 3-the number of mononucleolar cells in experiment with RNA; K'-the arithmetical mean of the number of mononucleolar cells for nonoperated animals; 4-the number of binucleolar cells in experiment with implantation of brain; 5-the number of binucleolar cells in experiment with administration of brain homogenate; 6-the number of binucleolar cells in experiment with RNA; K"-arithmetical mean of the number of binucleolar cells for nonoperated animals (Reznikov, 1965).

the implantation into the brain of brain pieces treated by 1% water solution of trypan blue. Mitoses were observed only in the damaged hemisphere, while amitotic divisions were present in equal amounts in the damaged and in the intact hemisphere. Prophases and metaphases were observed; anaphases and telophases of neurons were extremely rare (Fig. VII-13). Probably the mitoses of neurons were abortive. They were observed between the third and tenth days after the operation. During the same period mitotic divisions of glial cells were also observed (Fig. VII-14). Mitoses of neurons in the damaged large hemisphere cortex were described in rabbits and rats by a number of authors (Saltykow, 1905; Sui-Tsyn, 1959; Afanasyev and Kotovsky, 1959, 1961; Wenzel *et al.*, 1969, 1969a).

The injection of the preparations into the brain resulted in strong changes in the ependyma and subependymal layer. Their cells grew in size and became clearer (Fig. VII-15). They m

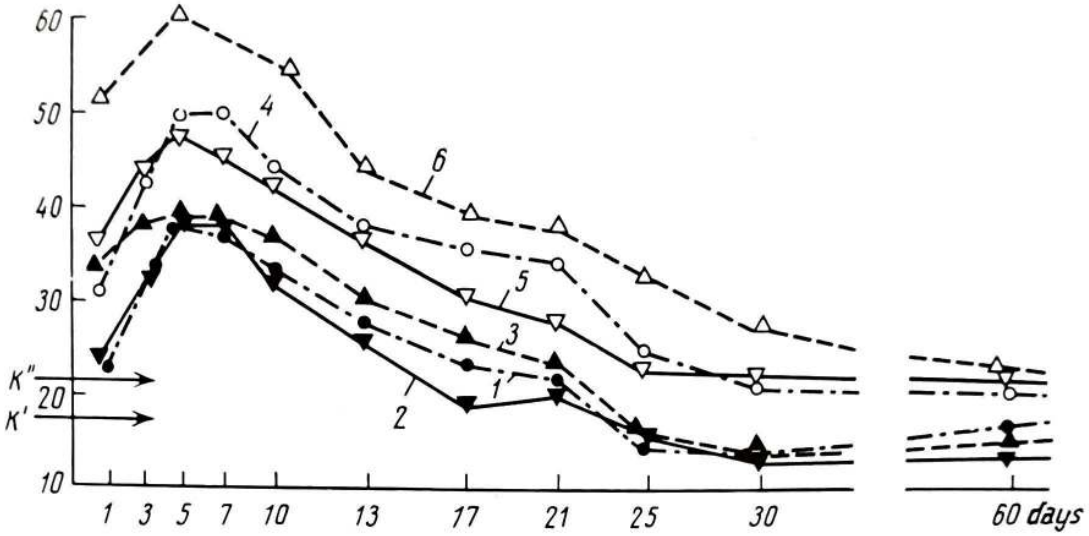


Figure VII-12. Dynamics of changes in number of binuclear and bicellular groups of nerve cells after administration of biostimulators into brain. 1-the number of binuclear cells in experiment with implantation of brain; 2-the number of binuclear cells after administration of brain homogenate; 3-the number of binuclear cells after administration of RNA; K'-arithmetical mean of the number of binuclear cells for nonoperated animals; 4-the number of bicellular groups after implantation of brain; 5-the number of bicellular groups after administration of homogenate; 6-the number of bicellular groups in experiment with RNA; K"-arithmetical mean of the number of bicellular groups for nonoperated animals (Reznikov, 1965).

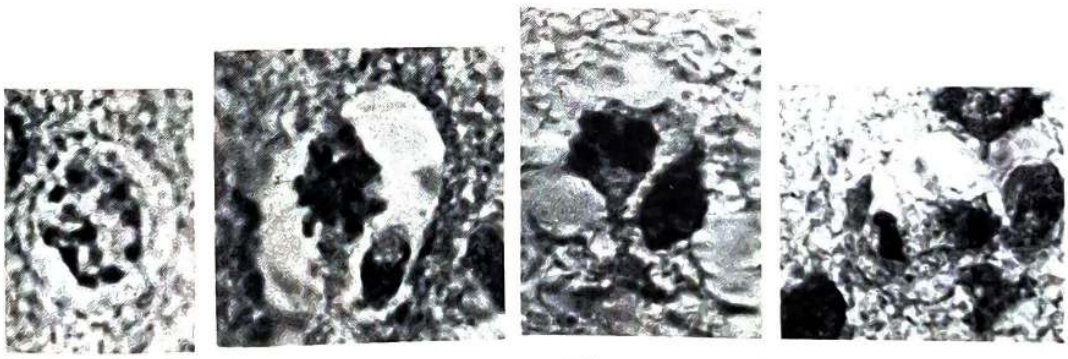


Figure VII-13. Mitoses of neurons. Three days after grafting of brain pieces into the cortex of cerebral hemispheres in rats. Ocular 10, objective 90. Left, Prophase; Center left, Me aphase; Center right, Anaphase; Right, Telophase.

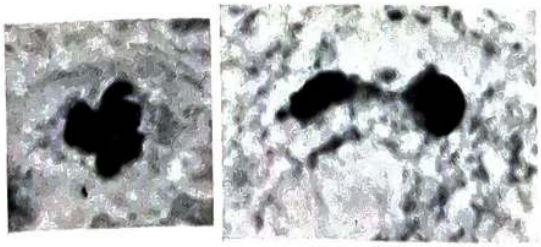


Figure VII-14. Mitoses of glya cells in the same experiment as Figure 13. Left, Metaphase. Right, Telophase.

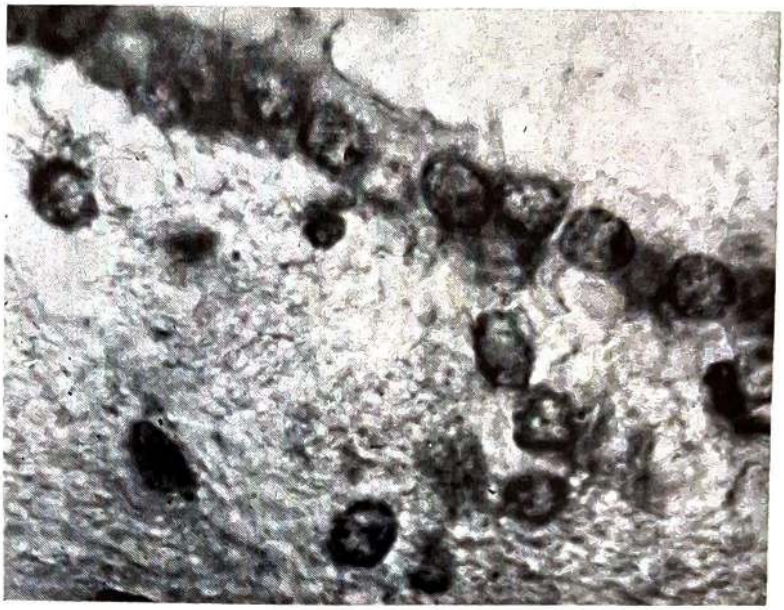


Figure VII-15. Ependyma of lateral ventriculum and wedging from it light cells entering white matter of cortex. Five days after grafting brain piece into brain. Ocular 7, objective 90.

Figure VII-16. Neurons in white matter of cortex of cerebral hemispheres far from ependyma in kitten. Three days after partial decortication. Ocular 7, objective 90.

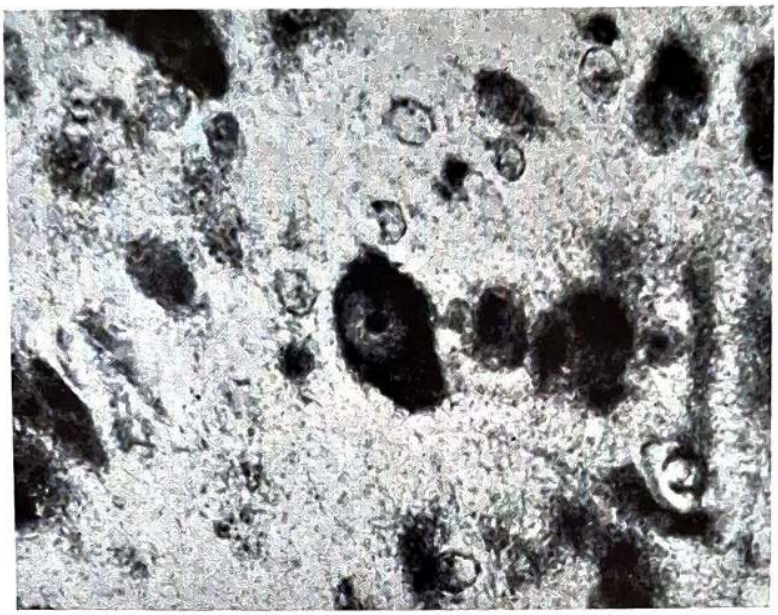


Figure VII-17. Formation of nerve cells in the white matter near ependyma in kitten. Three days after partial removal of the cortex. Ocular 7, objective 90.

grated into the white substance. It is remarkable that nerve cells also appear (Fig. VII-16,17). It can be suggested that some of these nerve cells are formed from the weakly differentiated cells, migrating from the subependymal layer.

Physiological investigation performed by the method of Schoen (1926) has shown that the injection of the preparations into the brain resulted in significant changes of sleep duration. The time necessary for the appearance of a reaction on the irritating factor in the case of a sleeping animal also was changed (Reznikov and Kolchin, 1966). In other words the injection of our preparations had regular and reproducible effects upon the intensity and relationship between excitatory and inhibitory processes.

The injection of the preparations into the brain leads to very pronounced effects upon the neurons. For example, the injection of RNA preparation into the rat brain resulted in a small polyploidization of nerve cells and increase of DNA content in their nuclei. This was shown by photometric measurement in the nuclei of layers II and III of the cortex (Fig. VII-18) (Reznikov, 1967). Damage of the brain in the adult mice or injection of RNA preparation into it stimulated DNA synthesis in glial cells, in subependymal layer and probably in small neurons of the large hemisphere cortex. This was concluded on the basis of autoradiographical investigation using H^3-thymidine (Reznikov, 1968) (Fig. VII-19).

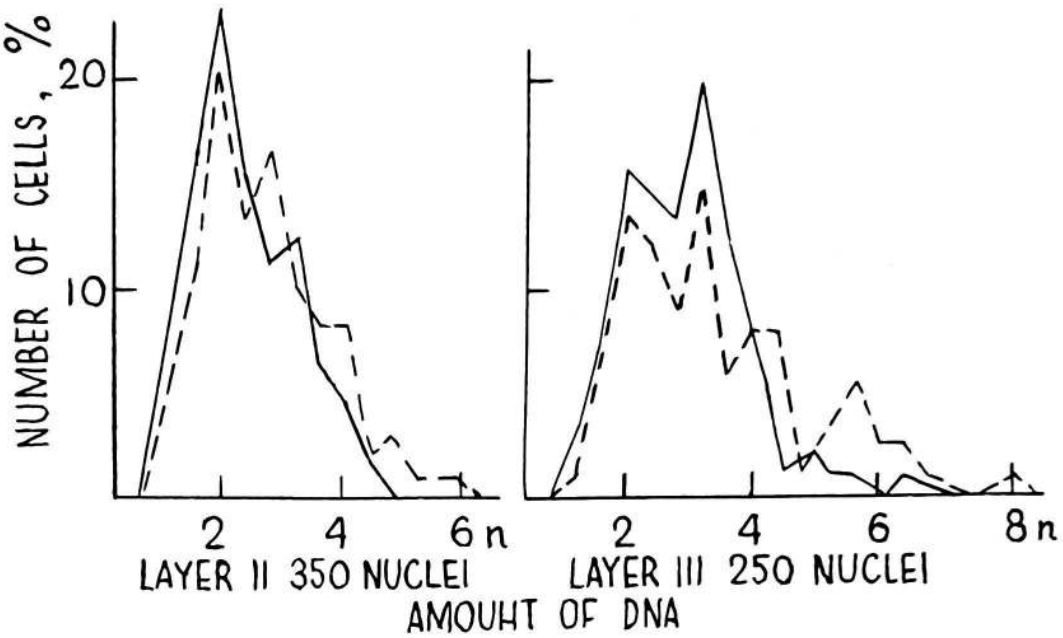


Figure VII-18. Variation curves of DNA content in nuclei of neurons in II and III layers of the cortex of cerebral hemispheres in brain of intact rats (left portion) and rats injected with RNA (right portion) (Reznikov, 1967).

Torskaya (1968, 1970) has shown, using caryometric, histochemical, autoradiographical and cytophotometrical methods, that distrophy and wearing out of 1.78-9.7% neurons takes place in the hippocampus of adult normal cats, rabbits and rats. Compensatory polyploidization of the remaining ones is an accompanying phenomenon. It involves mitotic and amitotic division of polyploid neurons, formation of endoprophases, as well as of binuclear and daughter neurons. Radioautographical experiments with H^3-thymidine have demonstrated occurrence of DNA synthesis in neurons (Fig. VII-20). In microregions of hippocampus labelling index, calculated per total number of neurons for a given region was as follows—for granular neurons 6.16% for pyramidal neurons 6.9%, for polymorphic neurons 15.73%. Trauma induced by the removal of certain brain region increased both atrophy of neurons and their division and polyploidization.

All these results lead to the following conclusions. Injection of certain biological preparations into the brain or into the liquor of the fourth ventricle stimulated processes of destruction and processes of restoration in the neurons of the brain cortex. In other words this stimulated the process of physiological regeneration of brain nervous tissue, the process of its restoration. This is accom-

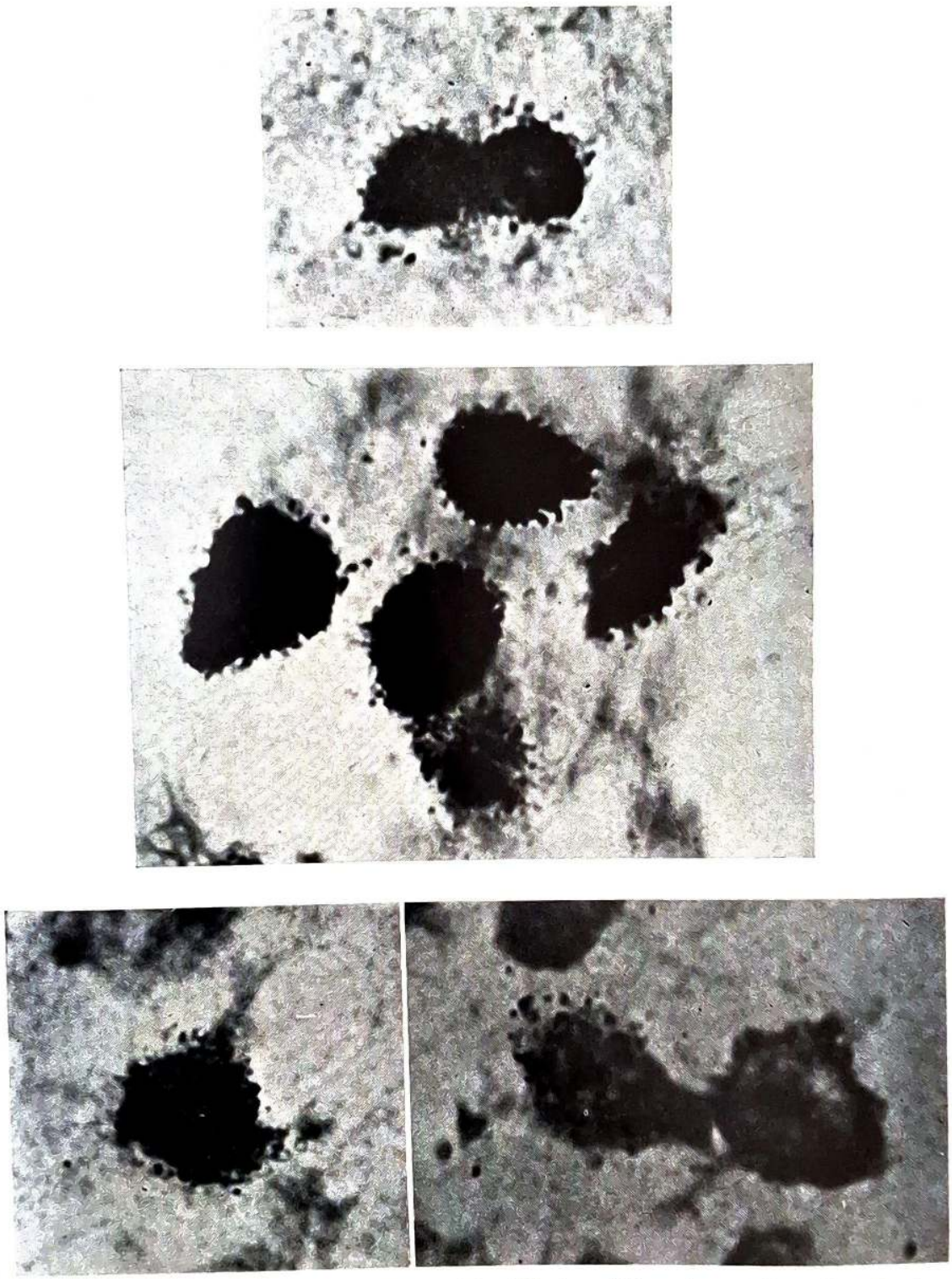


Figure VII-19. Cells labelled with H^3-thymidine in adult mice brain. Nissl staining. x 3500. Top, Dividing glyal cell. Top center, Cells of subependymal layer. Bottom, left and right, Apparently, nerve cells (Reznikov, 1968).

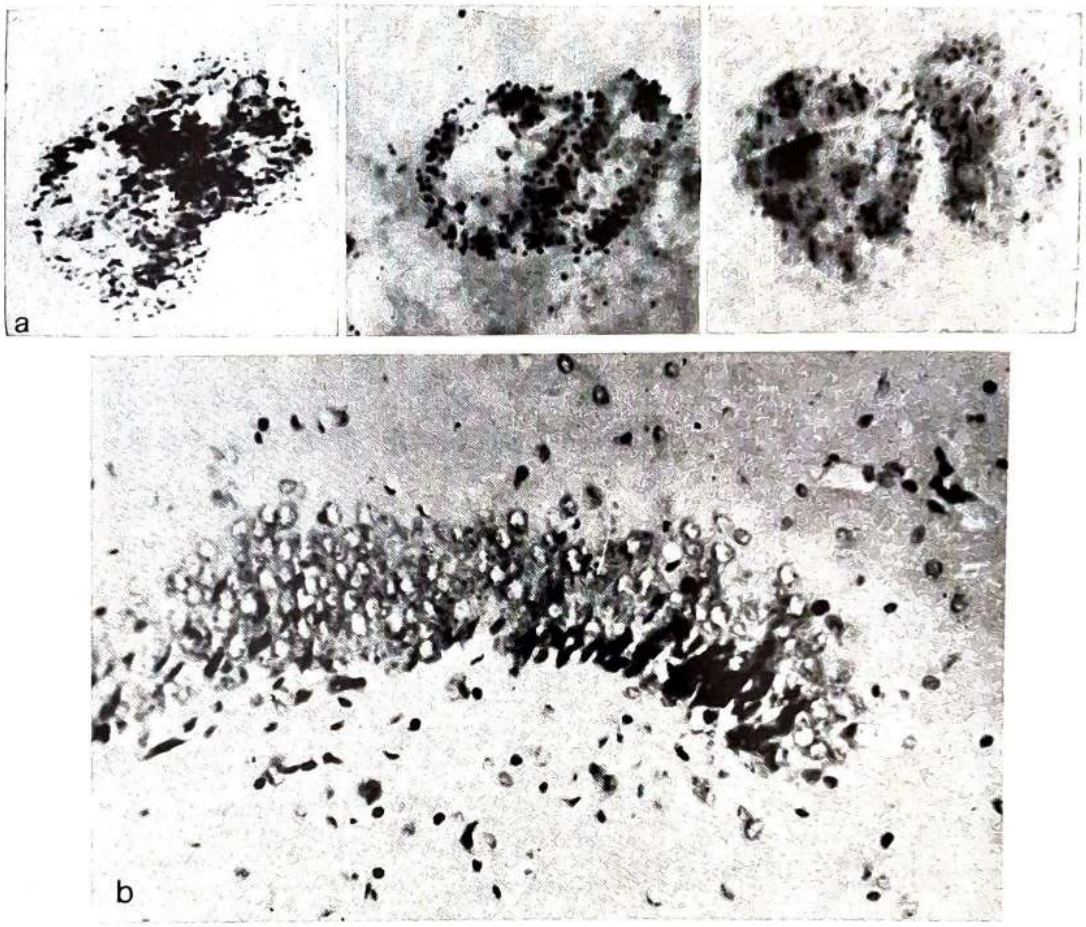
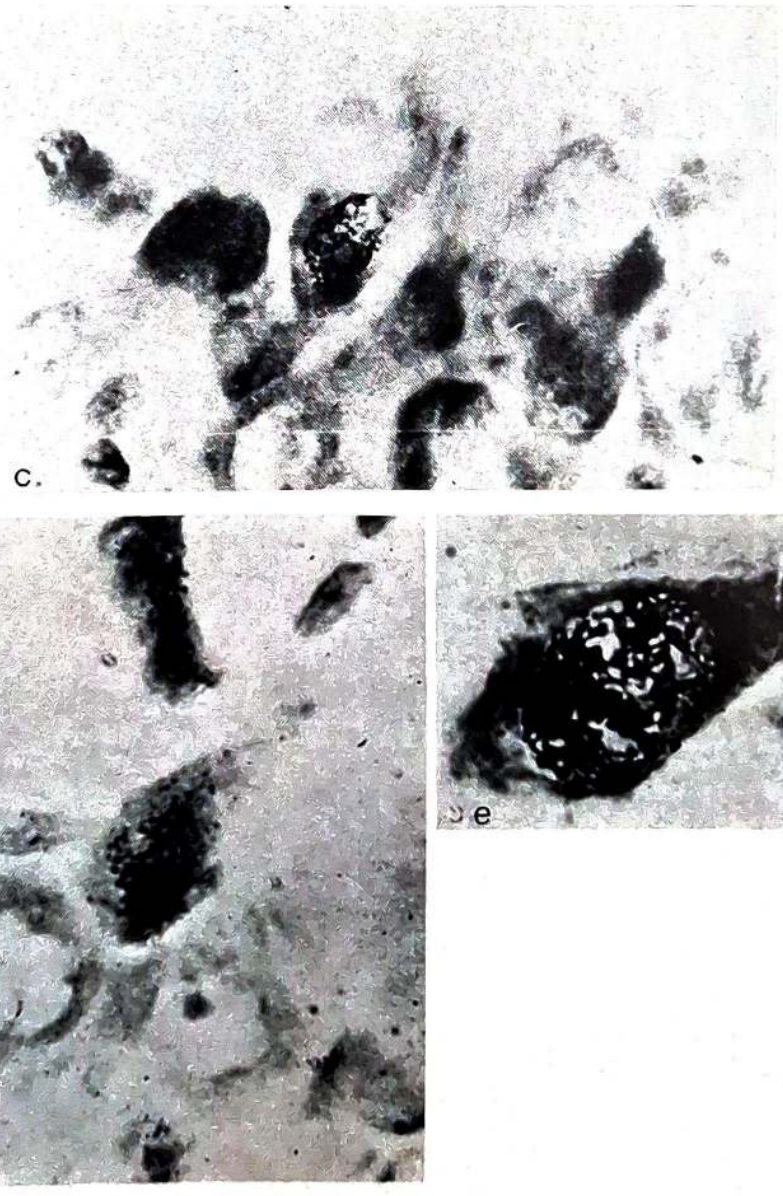
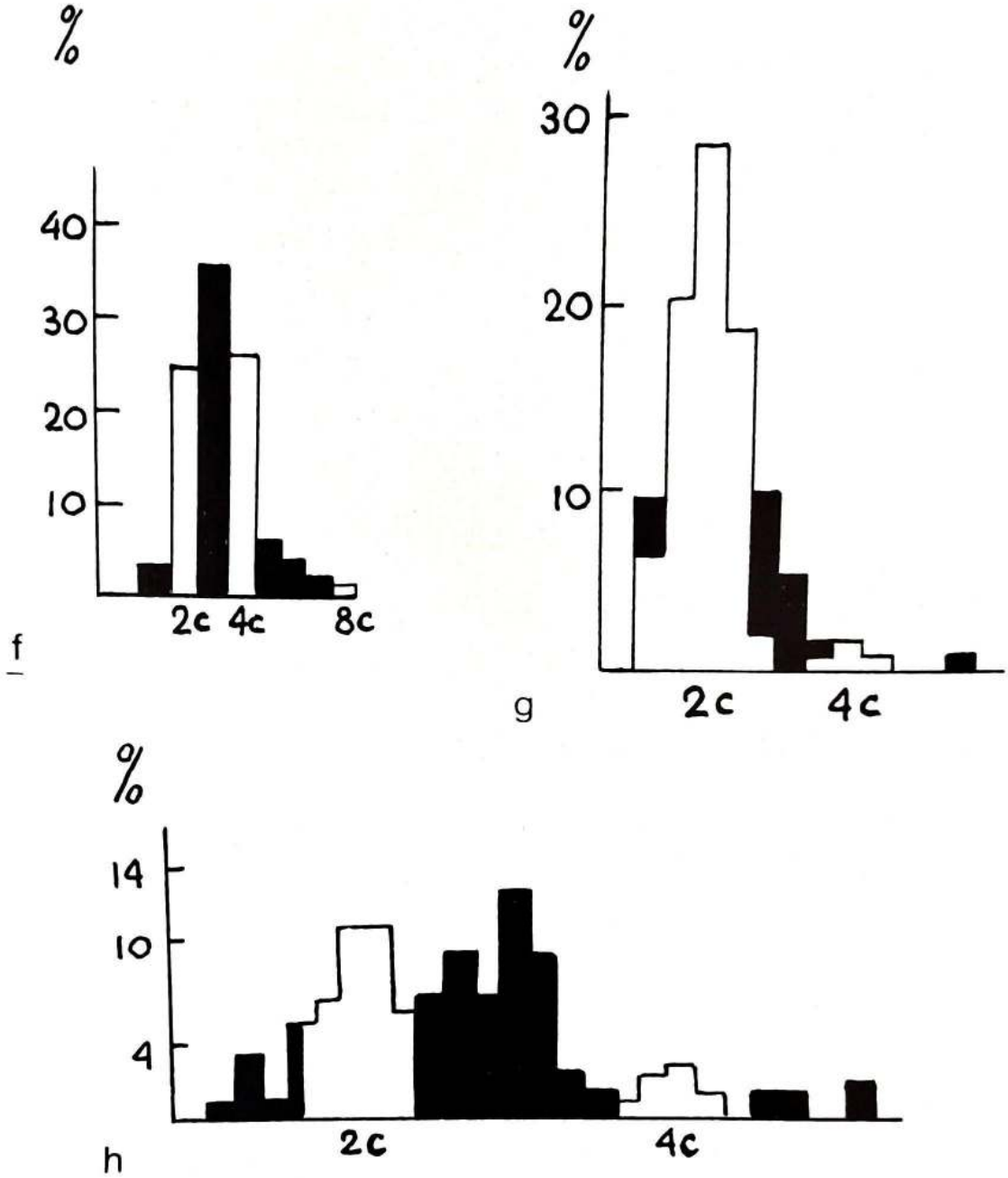


Figure VII-20. Division, DNA synthesis and poyploidization of hippocampal neurons in adult rats, mice, rabbits and cats (Torskaya, 1970). A. H^3-Thymidine label in amitotically dividing neuron nuclei in one-year-old mice. Hemalaum. x 1200. B. H^3thymidine label in the nuclei of fascia dentata granular neurons. Hematoxylin-eosin. x120. C. H^3-thymidine label in the endoprophase nucleus of pyramidal hippocompal neuron in the one-year-old rat on the 5th day after administration of H^3-thymidine. Hemalaum. x800. D. H^3-thymidine label in the polymorphic neurons on the 5th day after administration of H^3-thymidine. Hemalaum. x1200. E. Prophase on the two-thread stage. Large pyramidal cell. Feulgen-light green. x1200. F, G and H. Signature for the histogramms. White columns - nuclei classes containing 2n aliquot DNA amount. Black columns - nuclei classes containing DNA amount. Histogramma. The distribution of the nuclei of granular neurons according to the DNA contents. Fascia dentata of the hippocampus of the young rabbit (weight 1600 gm). G. Histogramma. The distribution of the nuclei of granular neurons according to the DNA contents. Fascia dentata of the hippocampus of the adult cat (weight 3600 gm). H. Histogramma. The distribution of the nuclei of granular neurons according to the DNA contents. Fifteen days after cortical ablation of contralateral hemisphere. Rabbit (weight 3600 gm).

panied by change of DNA synthesis in the neurons, molecular and structural rejuvenation of the nerve cells and it is reflected in the balance of excitatory and inhibitory processes in the central nervous system.

These experimental facts and the conclusions drawn from them appear to indicate a certain new perspective in the study of regeneration processes in the brain and this may present a certain interest for biology and medicine. On the other hand, these data show that only very little is done and further work in this direction is necessary.





Our brief essay presents only one of the approaches to the problem and describes some relevant facts. From our viewpoint they enable one to suggest a working hypothesis and its further development may lead to some results important both for theory and practice. It is our opinion, that not only firmly established concepts are important for the scientific research, but working hypotheses as well.

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VIII

GENERAL CONCLUSIONS

In the beginning of this book a question was put forward, whether in mammals and man one can induce regeneration of organs or tissues, which are not capable of regeneration under the usual conditions of their damage or removal. Or, saying it in other words can one achieve the recovery of ability to regenerate that is lost in the ontogenic and phylogenic development of these animals? Consideration of certain theoretical aspects of the problem and of experimental results leads to a positive answer to this question. It should be mentioned however that in some cases regeneration is not only stimulated, but one can actually recover the lost ability of mammalian organs and tissues to regenerate.

The loss of ability for organ-repair regeneration in ontogeny and phylogeny of animals is due to the decrease of ability to destruction and dedifferentiation of the basic tissues in these organs. In order to recover the lost regeneration ability, one must strongly stimulate the destruction and dedifferentiation of corresponding tissues. Evolutionary changes of ability to destruction and dedifferentiation on one hand and of the ability to repair regeneration on the other are considered to be a consequence of the adaptive changes of the organism and its metabolism to the changing environment. In order to recover the lost ability to regeneration one has to change the metabolism of a given organ, to make it more similar to the metabolism of a similar organ capable of regeneration.

The main trend of evolution in the animal kingdom was associated with the transition from aquatic to terrestrial way of life. In some groups of animals, such as tailless amphibia, for example, this transition is in some form reflected during their ontogeny—the tadpoles live in water while adult frogs live on land. This is accompanied by characteristic changes in the metabolism

of both the whole organism and its organs. The ability to repair regeneration is being lost. In axolotles, with their aquatic way of life the ability for regeneration is high. In lizards and rats with a terrestrial way of life it is low. By augmenting the destruction and dedifferentiation processes in the stump one can induce the regeneration of typical or atypical limbs or toes in late tadpoles, adult frogs, lizards, newborn rats or opossums. The recovery of the lost regeneration ability at early stages is easier than at late stages.

The ability to regenerate limbs in axolotles suppressed by x-ray irradiation can also be recovered. Irradiation practically does not affect the ability of tissues to destruction, but suppresses their capacity to dedifferentiation. In order to recover the regeneration ability suppressed by irradiation one has to augment tissue destruction and to stimulate and normalize altered nucleic acid and protein metabolism.

Stimulation of destruction and dedifferentiation may lead to complete regeneration of skull vault bones in dogs, rats and in man. These bones do not regenerate under usual damage conditions. Regeneration can be obtained by transplanting of specially prepared bone particles (dust) into the damaged region upon dura mater. The bone under these conditions regenerates by induction. Bone particles serve as inductor, cells of young immature connective tissues serve as reacting material, the presence of dura mater is a necessary prerequisite for the regeneration process.

In a similar way, regeneration of tooth tissues in dogs can be induced. These tissues do not regenerate under usual damage conditions. Dentin particles serve as in inductor, amphodont tissue represents the reacting material, regeneration of the dentin-like tooth tissue occurs via induction.

By augmenting of the destruction and dedifferentiation of tissues in the damaged region of heart muscle in adult rats, rabbits or dogs, by directed alteration of metabolism and uses of scarring inhibitors one can obtain regeneration of heart muscle fibers. The mechanism of this process is complex and obscure up to now. Heart muscle fibers can regenerate from the stumps of the fibers (but only to a limited extent) and on the other hand in the central part of the damaged region independently of the stumps. In the first case regenerated muscle fibers are histologically differentiated,

in the second case they can either be undifferentiated (in our experiments) or differentiated (in experiments of Sinitsyn). Products of heart muscle decomposition in the damaged area can serve as an inductor of heart muscle fibers regeneration. Cells of myogenic nature, originating from the stumps or possibly cells of some other origin serve as reacting material. These cells are transformed under the effect of a specified inductor. In this case metaplasia evoked by induction would be possible, as was the case in the experiments of Levander (1964). Regenerated heart muscle fibers showing no differentiation at the cytological level resolve after some time, those which are differentiated are retained much longer. If non-differentiated muscle fibers are formed by induction and metaplasia, then their incomplete differentiation and final resolution are easily understandable. If these elements are also used for the skull bone induction, then its good differentiation and long survival are also easily explained. The problem of heart muscle regeneration demands further studies.

The problem concerning the stimulation of nervous tissue regeneration is very complex but not hopeless. It is quite clear that peripheral nerves in mammals and man can regenerate and that transplantation of nerve pieces into the damaged region may stimulate nerve regeneration in recipients. It was shown that use of certain substances may selectively stimulate the growth of nerve processes, sensory and sympathic ganglia (Levi-Montalchini, 1965). The regeneration of the spinal cord in mammals can be achieved using the substances inhibiting glial scar formation alone or in combination with the substances stimulating axon regeneration (Windle, 1955; Nesmeyanova, 1968). The regeneration of the spinal cord is followed by the recovery of the lost locomotor function. And, at last, attempts were made to stimulate physiological regeneration of the brain cortex in mammals by injection of certain preparations into the brain or into the fluid of the fourth ventricle (Polezhaev, 1968). These treatments lead to molecular and structural rejuvenation of brain neurons and to the change in balance of exitatory and inhibitory processes in CNS. Binucleolar, binuclear and bicellular neurons were formed.

It is clear that only very little is done so far. Many points are not known and demand further investigation. But even at present

it is clear that the efforts in this direction may be rewarding, and this field is interesting from both theoretical and practical aspects of biology and medicine.

In conclusion, I would like to stress once again that due to limitations of space, insufficient amount of information on some questions, and the impossibility of analyzing thoroughly different aspects of the problem, I often presented only one personal viewpoint, which seemed to me most probable. This is particularly true for Chapters VI and VII as well as for a number of general points. It was not possible to present all arguments and to discuss the problem from various angles. Other more plausible and perfect explanations and concepts can be put forward to account for the outlined facts. The same is true with regard to experimental methods. Besides those, mentioned in this book, other methods of quantitative analysis, autoradiography, cytospectrophotometry etc. can be successfully applied. We think that the future studies will be enriched both by new ideas and by new methods. But the significance of the present investigation still will not be lost.

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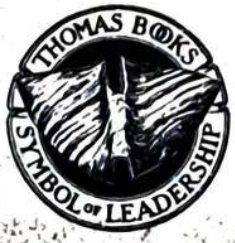

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